ALTERNATIVE THERAPEUTIC APPROACHES FOR MULTIDRUG RESISTANT CLOSTRIDIUM DIFFICILE

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PUBLISHED IN: Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-024-0
DOI 10.3389/978-2-88963-024-0

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ALTERNATIVE THERAPEUTIC APPROACHES FOR MULTIDRUG RESISTANT CLOSTRIDIUM DIFFICILE

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Clostridium difficile infection (CDI) is among the leading causes of infectious diarrhea among patients in hospitals. Multidrug resistance in C. difficile continues to plague antimicrobial chemotherapy of CDI, posing a major cause of concerns within healthcare and hospital environments. Hence, there is an urgent need for alternative therapeutic approaches for multidrug resistant C. difficile.

Citation: Janvilisri, T., Sorg, J. A., Scaria, J., Sadowsky, M. J., eds. (2019). Alternative Therapeutic Approaches for Multidrug Resistant Clostridium difficile. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-024-0

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frontiers in Microbiology

EDITORIAL
published: 31 May 2019
doi: 10.3389/fmicb.2019.01216

Editorial: Alternative Therapeutic Approaches For Multidrug Resistant *Clostridium difficile*

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Keywords: ***Clostridium difficile*, therapeutics, multidrug resistance, alternative therapy, antibiotics**

Editorial on the Research Topic

Alternative Therapeutic Approaches For Multidrug Resistant *Clostridium difficile*

OPEN ACCESS

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *05 February 2019*
Accepted: *15 May 2019*
Published: *31 May 2019*

Citation:
Janvilisri T, Sorg JA, Scaria J and Sadowsky MJ (2019) Editorial: Alternative Therapeutic Approaches For Multidrug Resistant Clostridium difficile. Front. Microbiol. 10:1216. doi: 10.3389/fmicb.2019.01216

Clostridium difficile infection (CDI) is among the leading causes of infectious diarrhea among patients in hospitals and is increasing in the community. Treatment with antibiotics, especially those with a broad spectrum of activities, disrupt normal intestinal flora and create a dysbiotic state that favor acquisition and proliferation of *C. difficile*. Therefore, antibiotic use is the primary risk factor for the development of CDI among hospitalized patients. The first lines of treatment for CDI include metronidazole and vancomycin. However, an emergence of hypervirulent strains, which are characteristically resistant to such antibiotics, contributes to an increase in numbers of CDI cases worldwide. Resistance to antibiotics in *C. difficile* alleviates effective chemotherapy of CDI. Furthermore, CDI patients might be more likely to pick up an infection, as *C. difficile* becomes more resistant and/or could spread resistances to other bacteria. The clinical impact of resistance is therefore immense, characterized by increased cost, length of hospital stay, and mortality, posing a major cause for concern within healthcare and hospital environments. Consequently, there is an urgent need for alternative therapeutic approaches to treat drug resistant *C. difficile*.

This Research Topic "Alternative Therapeutic Approaches For Multidrug Resistant *Clostridium difficile*" emphasizes various strategies for combating antibiotic resistance in *C. difficile,* including novel antimicrobials from different sources such as oligopeptides, small molecules, and herbal medicine. Therapeutic alternatives are also presented and include phage therapy, fecal transplantation, and microbiota restoration. This Research Topic area comprises two reviews, one mini review, and seven original research articles, representing a broad spectrum of experimental approaches and areas of investigation to address alternative measures to tackle drug resistance in *C. difficile*.

There are attempts to identify novel compounds for the treatment of CDI. A study by Yang et al. revealed that lauric acid, a medium-chain fatty acid, exhibited an inhibitory effect on *C. difficile* vegetative cell growth, spore outgrowth, and biofilm formation. It has been shown that the cytotoxic effect of this compound was mediated via production of reactive oxygen species and plasma membrane damage. Using a mouse model of CDI, the pre-treatment with lauric acid could reduce inflammation caused by *C. difficile* toxin. Another study by Kers et al. demonstrated that variants of Mutacin 1140, a lantibiotic produced by the Gram- positive bacterium *Streptococcus mutans*, could serve as a backbone for novel antimicrobials against *C. difficile*. The promising OG253, the

Phe_1Ile variant of Mutacin 1140, has been shown to be potent against *C. difficile in vitro*, with low toxicity to mammalian cells and high stability. Furthermore, the treatment with OG253 could prevent CDI relapse in a hamster model. In another study by Harnvoravongchai et al. asiatic acid, a triterpenoid from a tropical plant *Centella asiatica* was assessed for its potential as an antimicrobial alternatives against *C. difficile*. The compound exhibited bactericidal activity, potentially through cell membrane damage, without interacting with vancomycin and metronidazole. It was also shown to possess negative effects on cell motility. Altogether, these compounds could be further developed as alternative treatments to combat CDI.

Recently, the U.S. FDA approved three new antimicrobial agents against Gram-positive bacteria—Tedizolid, Dalbavancin, and Ceftobiprole. Binyamin et al. attempted to evaluate the *in vitro* activity of these three antibiotics against 84 strains of *C. difficile* using the E-test method. They found that dalbavancin and tedizolid could be potential therapeutic agents for the treatment of CDI. Furthermore, dalbavancin, which inhibits cell wall synthesis, was superior compared to the first-line drug vancomycin, and the beta-lactam ceftobiprole exhibited lower MIC compared to the third generation beta-lactam ceftriaxone. In a quest to search for antibiotic alternatives, Thanissery et al. developed an *in vitro* screening pipeline to evaluate molecules as potential non-antibiotic therapeutics for CDI. They showed that 2-aminoimidazole molecules, could inhibit the growth and toxin activity of *C. difficile*, without disrupting normal gut flora, although this compound does not interfere with *C. difficile* sporulation.

Antibody-based immunotherapies are currently under development for the treatment of CDI. In an excellent review article, Péchiné et al. established that targeting *C. difficile* surface components represents alternative strategies to combat CDI. They provided an overview of characterized *C. difficile* surface components and the host specific immune response. Comparative views on passive immunization with various types of antigens are explored. A large number of potential vaccine strategies to prevent or cure CDI and recurrences have also been discussed. Another example of the immunotherapy against CDI is given in a mini review by Forster et al.. The authors summarized how antibody-mediated therapy could be applied for treatment and prevention of CDI. They explored antibodies in the clinical development stage, that are given systematically including Actoxumab and Bezlotoxumab as well as orally such as a bovine antibody from hyperimmune colostral milk with their perspective on the effective employment as non-antibiotic interventions.

Over the past decade, a number of non-antibiotic approaches for CDI treatment have been proposed. In an outstanding review, Baktash et al. discusses the mechanistic insights in the success of fecal microbiota transplants (FMT) for the treatment of CDI. The rationale of using FMT against *C. difficile* is discussed with its possible effects on *C. difficile* life cycles, including colonization resistance by healthy microbiota, suppression of *C. difficile* spore germination and outgrowth by modulating bile acids. Bacteriophages have also gained tremendous attention as promising antibiotic alternatives against resistant bacteria. Phothichaisri et al. made an effort to isolate and characterize phages specific to *C. difficile*. A total of 5 temperate phages belonging to the Myoviridae family were identified. The authors demonstrated that one of the phages targeted the S-layer protein of *C. difficile* cell wall. These phages could therefore lead to development of novel therapeutic agents and detection strategies for *C. difficile*. It is also well known that colonization resistance is an effective method to combat CDI. Vedantam et al. developed two synthetic biologic agents, Syn-LAB 2.0 and Syn-LAB 2.1, which were derived from lactic acid bacteria, with a host-cell binding fragment of the *C. difficile* adhesin SlpA on their cell-surface. They demonstrated that both biologics were safe and tolerable in hamster and piglet models with high colonization rate and exhibited protective effects against CDI in animals. Thus, these synthetic biologics could be of interest for investigators and clinicians as an alternative resource for tackling *C. difficile*.

At present, the problems concerning antibiotic resistance are evident, especially in the case of CDI, where treatment with antibiotics is a risk factor for the disease. It will be a challenge to search for alternative measures against CDI. The editorial team hopes that this Research Topic will be useful for investigators in the field. Finally, we would like to thank the authors for their contributions in this Research Topic, and all the reviewers for their critical review of the manuscripts.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 17 January 2018
doi: 10.3389/fmicb.2017.02635

Lauric Acid Is an Inhibitor of *Clostridium difficile* Growth *in Vitro* and Reduces Inflammation in a Mouse Infection Model

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OPEN ACCESS

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *30 July 2017*
Accepted: *18 December 2017*
Published: *17 January 2018*

Citation:
Yang H-T, Chen J-W, Rathod J, Jiang Y-Z, Tsai P-J, Hung Y-P, Ko W-C, Paredes-Sabja D and Huang I-H (2018) Lauric Acid Is an Inhibitor of Clostridium difficile Growth in Vitro and Reduces Inflammation in a Mouse Infection Model. Front. Microbiol. 8:2635. doi: 10.3389/fmicb.2017.02635

Clostridium difficile is a Gram-positive, spore-forming anaerobic human gastrointestinal pathogen. *C. difficile* infection (CDI) is a major health concern worldwide, with symptoms ranging from diarrhea to pseudomembranous colitis, toxic megacolon, sepsis, and death. CDI onset and progression are mostly caused by intestinal dysbiosis and exposure to *C. difficile* spores. Current treatment strategies include antibiotics; however, antibiotic use is often associated with high recurrence rates and an increased risk of antibiotic resistance. Medium-chain fatty acids (MCFAs) have been revealed to inhibit the growth of multiple human bacterial pathogens. Components of coconut oil, which include lauric acid, have been revealed to inhibit *C. difficile* growth *in vitro*. In this study, we demonstrated that lauric acid exhibits potent antimicrobial activities against multiple toxigenic *C. difficile* isolates *in vitro*. The inhibitory effect of lauric acid is partly due to reactive oxygen species (ROS) generation and cell membrane damage. The administration of lauric acid considerably reduced biofilm formation and preformed biofilms in a dose-dependent manner. Importantly, in a mouse infection model, lauric acid pretreatment reduced CDI symptoms and proinflammatory cytokine production. Our combined results suggest that the naturally occurring MCFA lauric acid is a novel *C. difficile* inhibitor and is useful in the development of an alternative or adjunctive treatment for CDI.

Keywords: *Clostridium difficile*, medium-chain fatty acid, lauric acid, alternative therapy, natural product

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming bacillus that was first isolated from the gut of an infant and became medically important when it was found to be the leading cause of antibiotic-associated diarrhea (AAD) in hospital settings worldwide (Smits et al., 2016). It is estimated that 15–25% of AAD cases can be attributed to *C. difficile* infection (CDI) (Ananthakrishnan, 2011). Partly due to increased awareness and diagnosis, the incidence and economic burden of CDI

have increased yearly, and CDI cases have been reported in every continent (Burke and Lamont, 2014; Desai et al., 2016). Prior antibiotic exposure, advanced age (more than 65 years), prior hospitalization, the presence of an underlying illness, and proton pump inhibitor use have all been identified as risk factors for CDI (Vardakas et al., 2012; McDonald et al., 2015).

The principal virulence factors of *C. difficile* are two large cytotoxins, toxin A and toxin B, which have been reported to exhibit enterotoxigenic and cytotoxic activity (Pruitt et al., 2010; Abt et al., 2016). Both toxins are capable of severely inflaming the colon and disrupting the epithelial mucosal surface. According to the current guideline for CDI treatment, prior antibiotic retreatment should be discontinued and replaced with metronidazole as first-line treatment; vancomycin is administered for extremely severe cases or relapses (Surawicz et al., 2013). Recurrence, one of the hallmarks of CDI, is due to the ability of *C. difficile* to produce stress-resistant spores and partly due to the inability of the gut flora to be restored after antibiotic treatment; recurrence can occur in 25% of patients with CDI, and the rate can increase up to 40–60% following a second recurrence (Johnson, 2009). The recently Food and Drug Administration (FDA)-approved antibiotic fidaxomycin has been identified as having similar treatment effects as those of vancomycin while having a reduced impact on the gut flora (Louie et al., 2012), although the recurrence rate is still high. Several vaccines developed by pharmaceutical companies are currently being tested in clinical trials, but no active immunization therapies have been approved by the FDA. However, bezlotoxumab, an antitoxin B monoclonal antibody, has been approved for preventing CDI recurrence (Martin and Wilcox, 2016; Villafuerte Galvez and Kelly, 2017). Alternative treatment and preventive strategies against CDI are therefore required.

Various free fatty acids (FAs), as well as their monoglyceride derivatives, have long been known to exert antimicrobial effects on numerous bacterial pathogens (Galbraith et al., 1971; Kabara et al., 1972). Short-chain fatty acids (SCFAs) such as acetic, propionic, and butyric acid have been proven to exhibit antibacterial activity against various pathogens including *Vibrio parahaemolyticus*, *C. perfringens*, *Salmonella,* and *Helicobacter pylori* (Thompson and Hinton, 1997; Namkung et al., 2011; Immanuel et al., 2012; Yonezawa et al., 2012). Of all medium-chain fatty acids (MCFAs) tested *in vitro*, lauric acid (dodecanoic acid, C12:O) and capric acid (decanoic acid, C10:O) have been demonstrated to have the most potent effect against various bacterial, fungal, and viral pathogens (Kabara et al., 1972; Bartolotta et al., 2001; Bergsson et al., 2001; Rouse et al., 2005; Huang et al., 2011). Lauric acid, in particular, has been revealed to exhibit antibacterial activity against both Gram-positive and Gram-negative pathogens such as *Staphylococcus aureus, Streptococcus mutans, S. pyogenes, Escherichia coli, H. pylori*, and many others (Kabara et al., 1972; Rouse et al., 2005). A recent study demonstrated the bactericidal effects of MCFAs (caprylic, capric, and lauric acid) combined with edible plant essential oils (carvacrol, eugenol, β-resorcylic acid, *trans*-cinnamaldehyde, thymol, and vanillin) against *E. coli* O157:H7 (Kim and Rhee, 2016). As the primary FA of coconut oil is lauric acid (45–53%), it is of great interest to utilize coconut oil as a source of lauric acid. The antimicrobial properties of lauric acid, monolaurin, and their ester derivatives may be attributed to physicochemical processes as well as their interference with various cellular processes (Dayrit, 2015). Shilling et al. (2013) reported that MCFAs can inhibit the growth of the *C. difficile* strain ATCC 9689 *in vitro*, and that lauric acid exhibits the highest potency. However, the extent of and the mechanism by which lauric acid inhibits *C. difficile* and its destructive effects on bacterial physiology and the spore outgrowth ability have not been investigated comprehensively, and importantly, the effect of lauric acid on CDI *in vivo* has not been analyzed.

In the present study, we screened multiple FAs for their ability to inhibit *C. difficile* growth *in vitro* and confirmed that lauric acid exerts the highest inhibitory effect. A comprehensive analysis demonstrated that lauric acid could inhibit biofilm formation and reduce spore outgrowth. Mechanistic studies revealed that the inhibition of *C. difficile* was partly due to the generation of intracellular reactive oxygen species (ROS) and membrane damage. In a mouse infection model, lauric acid consumption decreased CDI-induced colon inflammation and diarrhea, supporting the hypothesis that lauric acid is a potential compound for CDI treatment.

MATERIALS AND METHODS

C. difficile Strains and Culturing Conditions

Eleven *C. difficile* strains were utilized in this study. Strains R20291 (ribotype 027, $tcdA^+$, $tcdB^+$, $tcdC^+$, $cdtA^-$, $ctdB^+$) and 630 (ribotypes 012, $tcdA^+$, $tcdB^+$, $tcdC^+$, $ctdA^-$, $ctdB^-$) used in this study are described elsewhere (McEllistrem et al., 2005; Buckley et al., 2011). The other nine isolates, which consisted of three tcdA+tcdB+ isolates (TNHP 29, 59, and 207), three tcdA−tcdB+ isolates (TNHP 79, 82, 403), and three tcdA−tcdB− isolates (1, 3, and 6), were originally isolated from a hospital in Southern Taiwan and have been described by Hung et al. (2016). All *C. difficile* strains were cultured anaerobically on brain–heart infusion (BHI) agar or in BHI broth (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 0.05% L-cysteine. Anaerobic experiments were conducted inside a Don Whitley DG250 anaerobic workstation (Don Whitley Scientific Ltd., West Yorkshire, United Kingdom).

Fatty Acid Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Half Maximal Inhibitory Concentration (IC_{50}) Determination

Fatty acids (propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid, octanoic acid, capric acid, lauric acid, myristic acid, and palmitic acid) were purchased from Sigma–Aldrich (St. Louis, MO, United States). A minimum inhibitory concentration (MIC) assay was conducted according to the guidelines of the Clinical Laboratory and Standards

Institute (formerly the National Committee for Clinical Laboratory Standards) for anaerobes. An overnight-grown culture was refreshed 50-fold in fresh brain–heart infusion-supplemented (BHIS) broth, incubated until the optical density at 600 nm (OD_{600}) was approximately 0.35, and then diluted 8-fold in 96-well microplates. FAs predissolved in dimethyl sulfoxide (DMSO) were then added to the bacterial suspension to reach a 5% DMSO final concentration. Concentrations of FAs ranged from 0.01 to 5 mg/mL. Bacterial cells were also incubated in BHIS + 1% DMSO only as a control. Plates were incubated anaerobically at 37°C for 24 h. To determine the MBC, bacterial suspensions from each well were streaked out onto BHIS agar plates and incubated for an additional 24 h. The MBC of each FA was defined as the lowest concentration at which no visible colony was observed. To determine the inhibitory effects of different concentrations of lauric acid on the *C. difficile* strain R20291, the percentage of growth at each concentration was calculated using the following equation: inhibition (%) = [1 − (OD_{600} of growth with lauric acid/OD_{600} of growth in broth only) × 100]. The growth inhibition rate was plotted against the log of the lauric acid concentration, and the IC_{50} value was defined as the value that caused a 50% reduction in bacterial growth. At least three independent samples were analyzed for each experiment.

Growth Curves of Various Concentrations of Lauric Acid Incubated with *C. difficile*

To determine the antibacterial activity of lauric acid on *C. difficile* growth, we employed different concentrations of lauric acid (1×, 4×, and 8× MBC) in the growth assay conducted in an anaerobic chamber. In the assay, 1% DMSO served as a treatment control. The *C. difficile* strain R20291 was cultured in BHIS broth at 37°C in the anaerobic chamber for 16 h, and the overnight cultures were then refreshed 50-fold in fresh BHIS broth until the late exponential to early stationary phase (OD_{600} = approximately 0.8). Next, bacterial suspensions were added to DMSO or lauric acid, and OD595 was determined using a Libra S2 Colorimeter (Biochrom, Cambridge, United Kingdom). At least three independent samples were analyzed for each experiment.

Biofilm Assay

To determine the effect of lauric acid on biofilm formation, an overnight culture of the *C. difficile* strain R20291 was refreshed to the late exponential to early stationary phase (OD_{600} = approximately 0.8) in BHIS broth and then diluted 100-fold in fresh medium (BHIS + 0.1 M glucose) in 24-well polystyrene plates. Lauric acid at concentrations ranging from 0.125× to 1× MBC was added, and plates were incubated anaerobically at 37°C for 72 h. To determine the effect of lauric acid on preformed biofilms, biofilms were prepared for 24 h before treatment with lauric acid at concentrations ranging from 1× to 4× MBC for another 24 h. Moreover, 20 μg/mL vancomycin [40× the MBC of strain R20291, (Dapa et al., 2013)] and 1% DMSO were used as controls. To quantify the biofilm mass, supernatants were carefully decanted, and biofilms that formed in all wells were allowed to dry at room temperature. Two percent crystal violet (CV) was added to each well for 30 min and then removed through methanol treatment for an additional 30 min. Extracted dye contents were quantified by measuring the absorbance at 595 nm by using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). Experiments were performed at least three times.

Spore Preparation

Spores were prepared by plating a 1:100 dilution of overnight culture onto BHIS agar plates and then incubating the plates for 10 days at 37°C under anaerobic conditions. Spores were harvested with ice-cold sterile distilled water and purified with 50% Nicodenz (Axis Shield, Oslo, Norway) using a previously described method (Sorg and Sonenshein, 2008). Spores were purified to >99% purity as determined using phase contrast microscopy, and the number of spores per milliliter was quantified through visual enumeration using a Neubauer Chamber (Sigma–Aldrich) prior to use.

Spore Germination and Outgrowth Assay

To monitor germination efficiency, purified spores were first heat activated by incubating them for 30 min at 60°C and were then adjusted to an OD_{600} of 1.0 in sterile water; 75-μL aliquots of spore suspensions were mixed with equal volumes of lauric acid or sterile water supplemented with 5% DMSO for 20 min at room temperature. Next, 10 mM taurocholic acid (Fisher Scientific) was added, and OD_{600} was measured at 2-min intervals (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific). The ratio of the OD_{600} at time X to the OD_{600} at time zero (t0) was plotted against time. The level of dipicolinic acid (DPA) release was monitored in real-time through terbium fluorescence, as described in previous studies (Bhattacharjee et al., 2015; Francis et al., 2015). Briefly, 75 μL of purified spores previously adjusted to the OD_{600} of 1.0 were resuspended in germination buffer (10 mM Tris-Cl, 150 mM NaCl, 100 mM glycine, pH 7.5) and were then treated with equal volumes of various concentrations of lauric acid for 20 min. Next, 10 mM taurocholic acid was added, and DPA release was monitored using a FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, United States) at an excitation wavelength of 270 nm and an emission wavelength of 545 nm. Spores boiled at 100°C for 30 min served as a positive control for total DPA release. Statistical analysis was performed using GraphPad Prism version 6.0. Three independent experiments were conducted. DPA release was calculated using the following equation:

$$\text{DPA release (\%)} = \frac{\text{RFU}_{\text{sample}}}{\text{Average of RFU}_{\text{boiled spores}}} \times 100\%$$

To measure spore outgrowth, from the aforementioned germination assay, a 100-μL aliquot of spores from each reaction was serially diluted (10^{-1}–10^{-7}) with sterile phosphate-buffered saline (PBS) and then spread onto BHIS agar plates supplemented with 0.l% sodium taurocholate (TA) and incubated anaerobically at 37°C overnight. Spore outgrowth was calculated using the

following equation:

$$\text{Spore outgrowth (\%)} = \frac{\text{CFU count}_{\text{lauric acid treated sample}}}{\text{Average of CFU count}_{\text{DMSO control}}} \times 100\%$$

Cytoplasmic Material Leakage Measurement

An overnight culture of *C. difficile* R20291 was diluted at 1:50 in fresh BHIS broth and then grown to an OD_{600} of approximately 0.8. Bacterial cells were then treated with various concentrations of lauric acid (1×–8× MBC) anaerobically at 37°C. Moreover, 1% DMSO in PBS served as the negative control, and 100 μg/mL nisin (Sigma–Aldrich) served as the positive control. At various time points, supernatants were collected, and the absorbances at 260 nm were recorded using a BioPhotometer UV/Visible Spectrophotometer (Eppendorf, Hamburger, Germany). Three independent experiments were performed.

Live/Dead Bacterial Viability Measurement

To measure cell viability, an overnight culture of *C. difficile* R20291 was diluted at 1:50 in fresh BHIS broth and then grown to an OD_{600} of approximately 0.8. Bacterial cells were then treated with lauric acid at 0.25× MBC or 1% DMSO in PBS for 20 min. Bacterial pellets were collected and resuspended in sterile PBS. Suspensions were then mixed with the LIVE/DEAD BacLight staining reagent mixture (Molecular Probes, Invitrogen) according to the manufacturer's instructions. Samples were visualized under a FluoView™ FV1000 confocal microscope (Olympus), and fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 500 nm (SYTO9) and 635 nm (propidium iodide). Cell viability is expressed as the ratio of SYTO-9-stained cells to the total number of cells. At least three independent trials were performed for each experiment.

Ultrathin-Section Transmission Electron Microscopy

Transmission electron microscopy (TEM) was employed to visualize the cells damaged by lauric acid treatment. *C. difficile* R20291 cells grown to the exponential phase were concentrated through centrifugation and treated with 0.25× MBC of lauric acid anaerobically for 15 min. Samples were embedded in Embed-812 (Electron Microscopy Sciences) and cut with an EM UC6 ultramicrotome (Leica, Wetzlar, Germany). Sections with a thickness of 90 nm were placed on copper grids (Electron Microscopy Sciences) and then stained with 2% uranyl acetate and lead citrate. Ultrathin sections were examined under a JEM-1400 transmission electron microscope (JEOL) with 120 kV acceleration and a 4k × 4k CCD Camera System Model 895 (Gatan, Inc.). The results are representative of three independent experiments.

Fluorescent Dye-Based Detection of ROS

Reactive oxygen species was measured using the carboxy derivative of fluorescein, CM-H2DCFDA (Life Technologies), according to the protocol provided by the manufacturer with the following modification: briefly, an overnight-grown *C. difficile* culture was refreshed to OD_{600} of approximately 0.8 in BHIS broth. Moreover, 198 μL of the bacterial suspension was incubated with 2 μL of stock CM-H2DCFDA anaerobically at 37°C for 30 min. Cells were then treated with 1× MBC of lauric acid for 10 min, and fluorescence was then measured using the FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The following controls were included: bacterial suspensions in BHIS broth containing 1% DMSO as the negative control; bacterial suspensions in BHIS broth containing 0.0035% hydrogen peroxide solution (H_2O_2; Sigma–Aldrich) and 10 mM tert-butyl hydroperoxide solution (TBHP; Sigma–Aldrich) as the positive control. The results are representative of three independent experiments.

Bacterial RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

An overnight culture of *C. difficile* R20291 was refreshed in Tryptone Yeast or BHIS broth and was grown anaerobically at 37°C until OD_{600} was approximately 0.8. Bacterial cells were treated with various concentrations of lauric acid or 1% DMSO (control group) for 30 min anaerobically at 37°C. Cells were harvested through centrifugation, and total RNA was isolated using the RNAprotect Bacteria Reagent (QIAGEN, Venlo, Netherlands) in accordance with the manufacturer's instructions. Genomic DNA was removed using RQ1 RNase-free DNase (Promega). RNA was reverse transcribed into complementary DNA (cDNA) by using SuperScript™ II Reverse Transcriptase (Invitrogen) and random primers (Thermo Fisher Scientific) according to the manufacturer's instructions. The relative transcriptional level of putative ROS-related genes between the control group and the lauric acid treatment group was measured through real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the 2x qPCRBIO SyGreen Mix Hi-Rox (PCR Biosystems) and gene specific primers (**Table 1**), according to the manufacturer's instructions. A StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method, with normalization to the reference gene 16s and the stated reference condition. Samples were analyzed in at least three independent trials. Statistical analyses were performed using GraphPad Prism 6.0.

An Animal Model of CDI

Specific-pathogen-free 8-week-old male C57BL/6 mice were housed in the Laboratory Animal Center of (NCKU). All mice were maintained and handled according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of NCKU. All animal studies were performed following the protocol

approved by the IACUC of NCKU (approval NCKU-IACUC-102-149) and the Biosafety and Radiation Safety Management Division of NCKU. The animal model of CDI was established as previously described (Chen et al., 2008; Liu et al., 2017; Pizarro-Guajardo et al., 2017). Five animals were administered 12 mg/kg (low dose) and 24 mg/kg (high dose) of lauric acid dissolved in PBS orogastrically once per day for 7 days prior to infection with *C. difficile* and once more 1 day following infection. To condition the animals for CDI, mice were fed drinking water containing an antibiotic mixture, which included 0.4 mg/mL vancomycin, 0.215 mg/mL metronidazole, 0.4 mg/mL kanamycin, 0.035 mg/mL gentamicin, and 850 U/mL colistin, for a total of 5 days before the challenge. All antibiotics were purchased from Sigma–Aldrich. On the day before the challenge, mice were fed the antibiotic mixture without vancomycin and metronidazole, which were excluded to avoid disrupting *C. difficile* colonization. Esomeprazole dissolved in PBS was given to all mice through oral gavage 12 h prior to infection (18.55 mg/kg) and immediately before infection (4.82 mg/kg). On the day of infection, 1×10^6 CFU of *C. difficile* R20291 spores were administered through oral gavage, and 4 mg/kg of clindamycin was injected intraperitoneally. Two days after infection, all animals were euthanized through CO_2 asphyxiation. The severity of diarrhea was scored in accordance with mice stool consistency, as follows: (0) well-formed pellets; (1) semiformed stools that did not adhere to the anus; (2) semiformed stools that adhered to the anus; and (3) liquid stools. Organs and gastrointestinal lavage (GAL) fluids were extracted for downstream analysis. The entire animal experiment was performed for a total of three independent sets, and representative results were obtained.

Fecal Colony-Forming Unit Determination

Fecal samples (premixed in PBS) were collected from the animals, immediately heat treated at 65°C for 20 min, and then serially diluted onto BHI agar containing 0.1% TA. Plates were incubated anaerobically at 37°C for 48 h, and colonies were counted for CFU determination.

Mice Colon RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Colon samples were extracted using RNeasy® Plus Mini kits (QIAGEN). RNA yield and quality were examined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with SuperScript™ II Reverse Transcriptase (Invitrogen, Waltham, MA, United States). The expression levels of proinflammatory cytokines and chemokines were measured through qRT-PCR using RealQ

TABLE 1 | Sequences of oligonucleotide primers used in this study.

Name	Sequence (5′–3′)	Species	Reference
16s-F	GAT TTA CTT CGG TAA AGA GCG G	*C. difficile*	This study
16s-R	CCT TAC CAA CTA GCT AAT CAG ACG	*C. difficile*	This study
TcdA-F	AAA GCT TTC GCT TTA GGC AGT G	*C. difficile*	This study
TcdA-R	CTC TAT GGC TGG GTT AAG GTG TTG	*C. difficile*	This study
TcdB-F	GAT CAC TTC TTT TCA GCA CCA TCA	*C. difficile*	This study
TcdB-R	AGC TTC TTA AAC CTG GTG TCC ATC	*C. difficile*	This study
CD1529-F	TGT CTT TGG TTC TGG TTG GG	*C. difficile*	This study
CD1529-R	ACT TAC AGG GCT ATC CTG ATT TG	*C. difficile*	This study
CD0757-F	GAC TTG TGG AAA CCT TGT AGG A	*C. difficile*	This study
CD0757-R	TGC TGC ATC TGT TGT ATT AGG A	*C. difficile*	This study
CD1716-F	CTG ACC CTG ACT TAG TTG CTA TAA A	*C. difficile*	This study
CD1716-R	ATA TGT CGC ACG TAC AAC TCC	*C. difficile*	This study
CD1465-F	GCT ATG CAA TAC TTG TCC CAA AG	*C. difficile*	This study
CD1465-R	GCT AAG CTC TTC TGC TGC TAT	*C. difficile*	This study
mβ-actin-F	ACT GCC GCA TCC TCC TCC TC	Mouse	Hung et al., 2015
mβ-actin-R	TGC CAC AGG ATT CCA TAC CC	Mouse	Hung et al., 2015
mTNF-α-F	CAT CTT CTC AAA ATT CGA GTG ACA A	Mouse	Hung et al., 2015
mTNF-α-R	TGG GAG TAG ACA AGG TAC AAC CC	Mouse	Hung et al., 2015
mIL-6-F	AGG ATA CCA CTC CCA ACA GAC	Mouse	Hung et al., 2015
mIL-6-R	GTG CAT CAT CGT TGT TCA TAC	Mouse	Hung et al., 2015
mIL-1β-F	GCA ACT GTT CCT GAA CTC AAC T	Mouse	Hung et al., 2015
mIL-1β-R	ATC TTT TGG GGT CCG TCA AT	Mouse	Hung et al., 2015
mMIP-2-F	TGT CAA TGC CTG AAG ACC CTG CC	Mouse	Hung et al., 2015
mMIP-2-R	AAC TTT TTG ACC GCC CTT GAG AGT GG	Mouse	Hung et al., 2015
mMCP-1-F	CCC ACT CAC CTG CTG CTA CT	Mouse	Hung et al., 2015
mMCP-1-R	TCT GGA CCC ATT CCT TCT TG	Mouse	Hung et al., 2015

Plus 2X Master Mix Green (Ampliqon, Denmark), with β-actin as the reference gene in each reaction (**Table 1**). The data were analyzed using the $\Delta\Delta Ct$ method and expressed as the fold change in the transcription level under the test condition compared with the average for the indicated control and were then normalized to the reference gene β-actin. Statistical analyses were performed using GraphPad Prism 6.0.

Cytokine and Chemokine Measurement

The concentrations of GAL cytokines and chemokines were measured using a DuoSet® enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions. Absorbance was measured at 450 nm using an iMark™ microplate reader (Bio-Rad, Hercules, CA, United States). Samples were measured in triplicate, and statistical analyses were performed using GraphPad Prism version 6.0.

Statistics

All data are expressed as the mean ± standard deviations of at least three independent experiments. Statistical comparisons among the groups were made using Student's *t*-test. Multiple intergroup comparisons were made using one-way analysis of variance (ANOVA), followed by a *post hoc* Tukey's test with GraphPad Prism version 6.0. Statistical significance was set at $P < 0.05$.

RESULTS

Antibacterial Activity of Free FAs against *C. difficile* R20291

To identify the free FAs with potent *C. difficile* inhibitory effects, FAs C3–C16 were coincubated with log-phase-grown *C. difficile* cells, and their MBCs were determined (**Table 2**). For the test strain R20291, butyric acid (MBC = 50 mg/mL) showed the least inhibitory effects of all FAs tested, whereas lauric acid (MBC = 0.3125 mg/mL) showed the most potent effects. The length of the carbon chain did not appear to be an influencing factor contributing to the antibacterial activity of the FAs, although remarkably MCFAs appeared to exhibit lower MBCs. Specifically, the MBCs of both capric acid (C10) (1.25 mg/mL) and lauric acid (C12) (0.3125 mg/mL) were the second lowest and the lowest of all FAs tested. Moreover, the resulting pH variation in BHIS medium did not appear to contribute to the antibacterial activity of the various FAs (**Table 2**). As the MBC of lauric acid was significantly lower (fourfold, $P < 0.0001$) than that of capric acid, lauric acid was chosen as a potential candidate for subsequent experiments.

To further confirm the inhibitory effect of lauric acid on *C. difficile* growth, 10 additional clinical isolates of different toxinotypes were subjected to the same experiment (**Table 3** and **Figure 1**) (Hung et al., 2016). The MICs of all 11 tested isolates ranged from 0.08 to 0.16 mg/mL; however, the MBCs were all 0.31 mg/mL. As the MBCs were all the same, we assumed the inhibitory effect of lauric acid on *C. difficile* is likely not strain-dependent; hence, all subsequent experiments were performed using the laboratory strains 630 and R20291. To gain further insight into the inhibitory effect of lauric acid on *C. difficile*, the IC_{50} was determined. As depicted in **Figures 2A,B**, the IC_{50} of lauric acid against *C. difficile* strains R20291 and 630 was 12.48 and 33.67 μg/mL, respectively. The antibacterial activity of lauric acid against strain R20291 was further evaluated in liquid nutrient broth containing different concentrations of lauric acid (**Figure 2C**). When lauric acid was applied at 2× and 4× MBC, cell lysis appeared to occur immediately, as evidenced by the drop in OD. In short, lauric acid was revealed to display an inhibitory effect on multiple strains of *C. difficile*, and the inhibition mechanism is likely bactericidal.

Effects of Lauric Acid Treatment on Biofilm Formation and Stability

To further understand the effect of lauric acid on *C. difficile*, we determined whether lauric acid treatment affects biofilm formation and stability. In this study, we measured the effect of lauric acid treatment on biofilm formation by R20291 and 630 strains, as these two strains were reported to exhibit different biofilm forming abilities (Dapa et al., 2013). Clinical *C. difficile* strains are known to form robust biofilms *in vitro*, and these biofilm-dwelling cells are more resistant to antibiotics and perhaps to even host defenses than planktonic cells are (Dapa and Unnikrishnan, 2013; Dapa et al., 2013; Crowther et al., 2014). A previous study reported that vancomycin applied at 20 μg/mL (100× MIC) can significantly reduce the survival of *C. difficile* biofilm cells (Dapa and Unnikrishnan, 2013). For the strain R20291, vancomycin applied at 100× MIC could reduce biofilm formation by 15.5-fold compared with the DMSO control ($P < 0.0001$). However, 0.25× MBC of lauric acid could significantly reduce biofilm formation by 24.9-fold compared with the control ($P < 0.0001$) (**Figure 3A**). Similarly, for the strain 630, although vancomycin treatment led to a 20.8-fold reduction in biofilm formation, 0.25× MBC of lauric acid led to a 47.2-fold reduction (**Figure 3B**). To determine whether lauric acid treatment also disrupts established biofilms, a static culture of *C. difficile* was grown in multi-well plates for 24 h before lauric acid addition. Interestingly, although vancomycin applied at 100× MIC did not reduce the biofilm mass, lauric acid at least 2× MBC and 1× MBC could reduce the mass of biofilms formed by strains R20291 and 630, respectively (**Figures 3C,D**). Reduction of the mass of biofilms formed by the strain R20291 required a higher concentration of lauric acid than that from strain 630.

Effect of Lauric Acid Treatment on Spore Germination and Outgrowth

In addition to biofilm formation, the spore-forming ability of *C. difficile* contributes to its transmission (Deakin et al., 2012). Spores are known to be resistant to multiple environmental stresses, including cold, heat, desiccation, antiseptics, and antibacterial products (Rodriguez-Palacios and Lejeune, 2011;

TABLE 2 | Antibacterial activity of fatty acids against *C. difficile* strain R20291.

	Number of carbon backbone	General name	MIC (mg/ml)	MBC (mg/ml)	pH (in BHIS)
SCFAs	C3	Propionic acid	1.25	5	3.91
	C4	Butyric acid	25	50	7.11
	C4	Isobutyric acid	1.25	5	4.97
	C5	Valeric acid	1.25	2.5	5.78
	C5	Isovaleric acid	2.5	2.5	5.76
MCFAs	C6	Hexanoic acid	1.25	2.5	5.94
	C8	Octanoic acid	2.5	5	5.51
	C10	Capric acid	0.63	1.25	6.81
	C12	Lauric acid	0.31	0.31	6.91
LCFAs	C14	Myristic acid	>10	>10	6.51
	C16	Palmitic acid	10	10	6.77

Clostridium difficile strain R20291 was exposed to various dilutions of different fatty acids for 24 h, and MBC was determined as the concentration of fatty acids at which no visible growth was observed. Results are expressed as average of at least three independent replicates. SCFA, short-chain fatty acids; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids.

TABLE 3 | Inhibition of *C. difficile* strains by lauric acid.

C. difficile strain	Toxin genotype	MIC (mg/ml)	MBC (mg/ml)	Ribotype
R20291	*tcdA+tcdB+* (laboratory strains)	0.08	0.31	RT 027
630		0.08	0.31	RT 012
TNHP 20	*tcdA+tcdB+* (clinical isolate strains)	0.16	0.31	RT 002
TNHP 59		0.08	0.31	RT 002
TNHP 207		0.16	0.31	RT 106
TNHP 79	*tcdA-tcdB+* (clinical isolate strains)	0.08	0.31	RT 017
TNHP 82		0.08	0.31	RT 017
TNHP 403		0.16	0.31	RT 017
TNHP 1	*tcdA−tcdB−* (isolated from asymptomatic adults)	0.08	0.31	ND
TNHP 3		0.16	0.31	ND
TNHP 6		0.16	0.31	ND

Clostridium difficile isolates containing two laboratory strains and nine local isolates were exposed to various concentration of lauric acid for 24 h. MBC was determined as described previously. Results are expressed as average of at least three independent replicates. ND – not determined.

Deng et al., 2015; Edwards et al., 2016), and they are therefore a critical component of the pathogenesis of CID. One of the key germinating signals for *C. difficile* spores is the presence of TA. In the presence of TA, spores will undergo core hydration, and this process can be visualized microscopically; previously dormant phase-dark spores will become phase-bright due to core hydration and the eventual degradation of the cortex peptidoglycan. To measure the effect of lauric acid on spore germination, purified *C. difficile* strain R20291 spores were treated with taurocholic acid and various concentrations of lauric acid. As revealed in **Figure 4A**, in the presence of TA, the OD_{600} of the spore suspension decreased significantly within 20 min of exposure (PC group, $P < 0.0001$), whereas the DMSO control remained phase-dark (NC group). Interestingly, when various concentrations of lauric acid were added along with TA, the decrease in OD_{600} was faster over time than that in the PC group, suggesting that core degradation occurred at a faster rate ($P < 0.0001$ for all concentrations compared with the PC group). We also performed the same assay in the absence of TA, and no significant decrease in OD_{600} was observed for all concentrations of lauric acid tested throughout the 20 min of observation, suggesting that the effect was TA-dependent (**Figure 4B**). During spore germination, DPA release can be measured and is often used as a sign of spore germination. To further investigate the role of lauric acid in spore germination, we measured DPA released for 20 min (**Figure 4C**). As expected, DPA released from boiled spores was detected by its high fluorescence signals, which were higher than those of non-treated spores. Similarly, in the presence of TA, an increase in the fluorescent signal was detected, suggesting that DPA release was initiated at approximately 4 min and continued to increase until the end of the experiment. However, the addition of lauric acid, regardless of concentration, did not significantly alter DPA release over the course of the experiment ($P > 0.9999$). Furthermore, the addition of lauric acid alone did not induce any DPA release (data not shown). Finally, the viability of spores in the presence of lauric acid was measured (**Figure 4D**). Compared with spores exposed to TA only, lauric acid treatment in the presence of TA considerably decreased the rate of spore outgrowth in a dose-dependent manner. Spore outgrowth decreased to 64.5, 61, 60.4, and 39.6% of typical growth when subjected to lauric acid concentrations of 0.5× MBC, 1× MBC, 2× MBC, and 4× MBC, respectively (**Figure 4D**). These combined results demonstrate that lauric acid

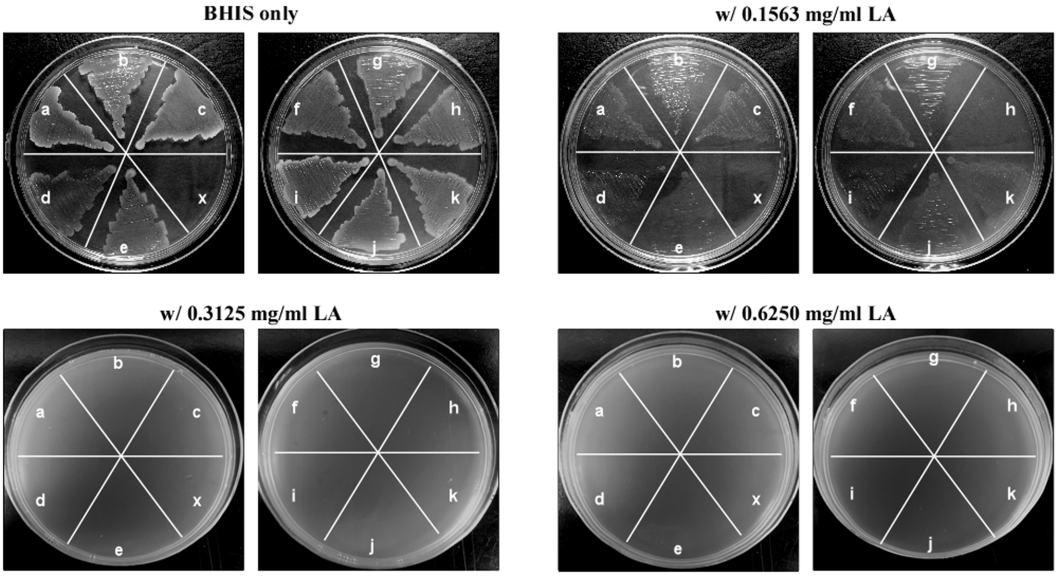

FIGURE 1 | Lauric acid is a potent inhibitor of multiple *Clostridium difficile* clinical isolates. Growth inhibition of multiple *C. difficile* isolates on BHI agar plates containing lauric acid at 0.5× (0.1563 mg/mL), 1× (0.3125 mg/mL), and 2× (0.625 mg/mL) MBC. a: TNHP 207; b: TNHP 59; c: TNHP 20; d: 630; e: R20291; f: TNHP 403; g: TNHP 82; h: TNHP 79; i: TNHP1; j: TNHP 3; k: TNHP 6; x: negative control. Results are representative of at least three independent experiments.

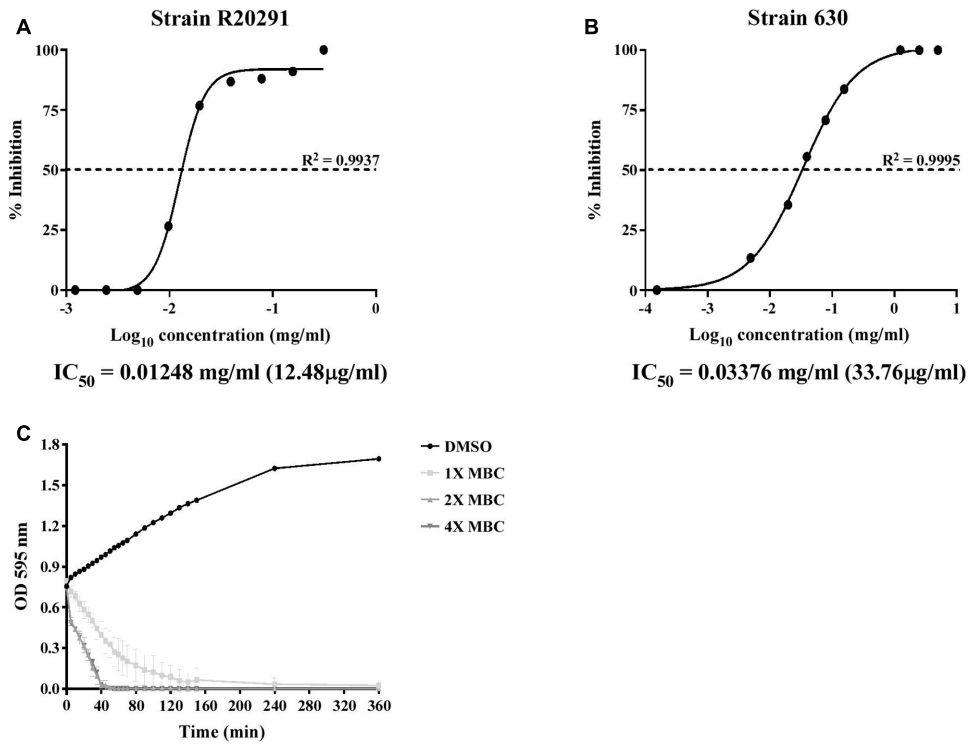

FIGURE 2 | IC_{50} determination and time-dependent antibacterial kinetic curve of lauric acid against *C. difficile*. **(A)** *C. difficile* strains R20291 and 630 were treated with various concentrations of lauric acid for 24 h, and the OD at 600 nm was then measured. Growth inhibition was normalized to the 5% DMSO control group. **(B)** Log-phase grown *C. difficile* R20291 cells were incubated with various concentrations of lauric acid in BHI broth for up to 6 h. **(C)** *C. difficile* R20291 cells were incubated with 1×, 2×, and 4× MBC of lauric acid, and growth was monitored over time by measuring OD595. DMSO was included as a control. Results are expressed as mean of triplicate samples at least three independent experiments.

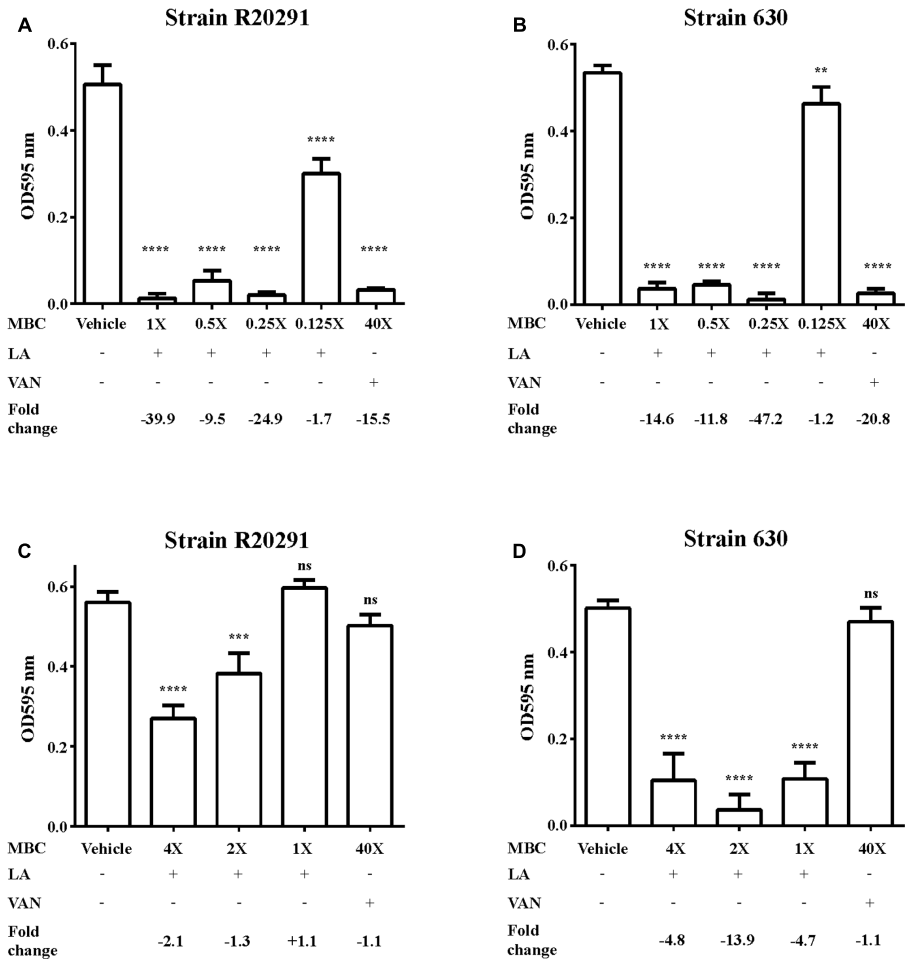

FIGURE 3 | Inhibition of *C. difficile* biofilm by lauric acid. Various concentrations of lauric acid were added to strains R20291 **(A)** and 630 **(B)** grown in multi-well plates, and adherent biofilms were quantified by CV staining. The effect of lauric acid on preformed biofilms was measured by incubating various concentrations of lauric acid with a 24-h old biofilm formed by strains R20291 **(C)** and 630 **(D)** for an additional 24 h. The disruption of the preformed biofilm was quantified through CV staining. Vehicle: 1% DMSO only. LA: lauric acid. VAN: 20 μg/mL vancomycin (40× MBC). Lauric acid MBC: 4× = 1.25 mg/mL, 2× = 0.63 mg/mL, 1× = 0.31 mg/mL, 0.5× = 0.16 mg/mL, 0.25× = 0.08 mg/mL, 0.125× = 0.04 mg/mL. Results are the mean of triplicate samples, and one-way ANOVA was performed to assess significance. (ns, no significance; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

treatment can inhibit *C. difficile* biofilm formation and stability and can affect spore outgrowth.

Lauric Acid Inhibited *C. difficile* by Inducing ROS Generation and Cell Membrane Damage

To determine whether the inhibitory effect of lauric acid on *C. difficile* growth is due to the disruption of cell membrane integrity, *C. difficile* cells were treated with lauric acid, and the extracellular presence of released nucleic acid was measured (**Figure 5A**). In this assay, we included nisin, a broad-spectrum polycyclic antibacterial peptide produced by *Lactococcus lactis*, as a positive control, as it is known to attack bacterial cell membranes; this leads to cytoplasmic content release and cell lysis eventually (Ruhr and Sahl, 1985; Nobmann et al., 2010). As expected, in the presence of the antibacterial peptide nisin, a significant quantity of nucleic acids was detected in the culture supernatant at 30 min after treatment. When cells were treated with various concentrations of lauric acid, a considerably higher quantity of nucleic acid materials was also detected compared with the negative control, indicating that the addition of lauric acid compromised cell membrane integrity. The viability of *C. difficile* cells treated with lauric acid was further assessed using LIVE/DEAD staining and was visualized using confocal microscopy (**Figure 5B**). As lauric acid induced the rapid lysis of *C. difficile* cells, as indicated in **Figure 2C**, to elucidate the effects of lauric acid on *C. difficile* cells, these cells were treated with sublethal concentrations of lauric acid (0.25× MBC). When cell viability was quantified based on the percentage of cells that stained positive for propidium iodide, treatment with lauric acid at 0.25× MBC decreased viability to approximately 65% by 15 min after treatment (**Figure 5C**). By 30 min after treatment, the viability decreased to approximately 35%. By contrast, the

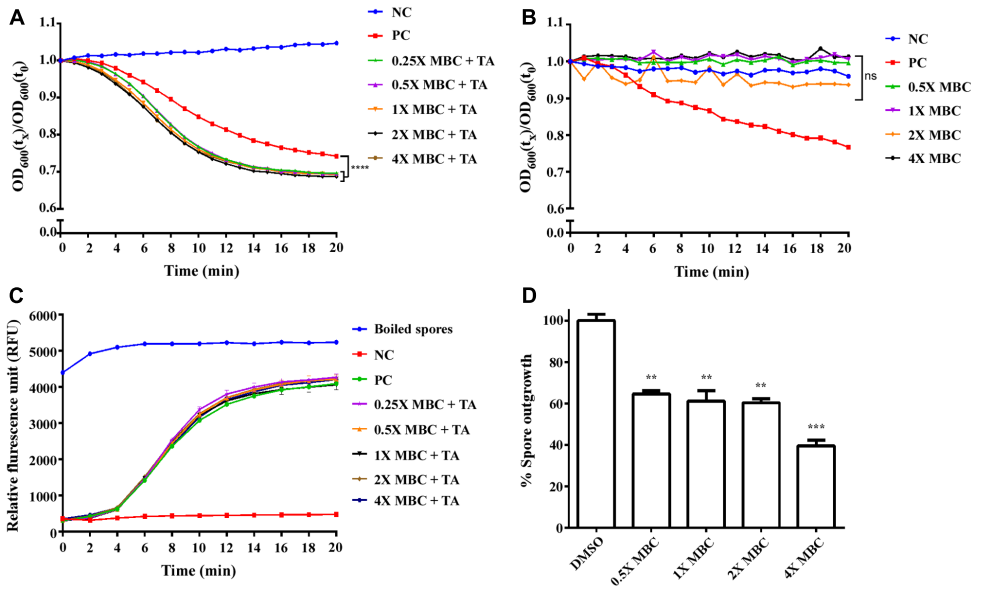

FIGURE 4 | Effect of lauric acid on *C. difficile* spore germination. Heat-activated spores from the *C. difficile* strain R20291 were incubated with 10 mM sodium taurocholate plus 5% DMSO (positive control, PC), 5% DMSO only (negative control, NC), or various concentrations of lauric acid plus 10 mM sodium taurocholate and 5% DMSO. Germination was monitored by measuring absorbance at 600 nm **(A,B)** or DPA release **(C)**. **(D)** Spore outgrowth was assayed by incubating spores in the presence of various concentrations of lauric acid for 20 min, and CFU/mL was determined by plating aliquots of spores onto BHIS agar containing TA. Results are the mean of triplicate samples, and one-way ANOVA was performed to assess significance. (ns, no significance; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

viability of the DMSO-treated control remained relatively high (15 min: 87%, 30 min: 84%). Ultrathin-section TEM analysis revealed that 20 min of lauric acid treatment (0.25× MBC) was sufficient to induce substantial cell death, as indicated by abnormal cell morphology and cytoplasmic content leakage. These findings are similar to those of Shilling et al. (2013) (**Figure 5D** and Supplementary Figure 1).

To determine whether lauric acid also induces ROS generation, vegetative *C. difficile* R20291 cells were treated with a sublethal concentration of lauric acid (0.25× and 0.5× MBC) for up to 60 min, and intracellular ROS levels were measured (see section "Materials and Methods"). As depicted in **Figure 6A**, treatment with the antiseptic hydrogen peroxide and tert-butyl hydroperoxide (TBHP) generated a considerable level of ROS in a time-dependent manner. Interestingly, lauric acid treatment also generated a substantial level of ROS over the course of the experiment. As no molecular or biochemical studies have been performed in *C. difficile* on ROS-associated genes, from the genomic annotation, we selected four potential antioxidant defense-associated genes. As depicted in **Figure 6B**, in the presence of 0.25× MBC of lauric acid, the expression levels of genes encoding for a putative superoxide dismutase (locus tag CDR20291_1529) and a putative catalase (locus tag CDR20291_1465) were upregulated (9.1- and 3.4-0 fold, respectively, compared with untreated cells) (**Figure 6B**). However, the gene expression levels of CDR20291_C0757, which encodes for a putative superoxide reductase, and CDR20291_1716, which encodes for a putative peroxidase, were not significantly altered in the presence of either 0.25× or 0.5× MBC of lauric acid. Nevertheless, the results of these experiments suggested that the bactericidal activity of lauric acid was partly due to the induction of membrane damage and ROS generation, which resulted in cell lysis.

Lauric Acid Pretreatment Decreased *C. difficile*-Induced Inflammation in a Mouse Infection Model

Finally, to determine whether lauric acid affects CDI *in vivo*, C57BL/6 mice were administered lauric acid orogastrically for 1 week prior to infection with purified *C. difficile* R20291 spores (Supplementary Figure 2). Mice administered only PBS prior to infection displayed symptoms of CDI, including a lack of well-formed feces due to diarrhea and a considerable decrease in body and cecum weight (**Figures 7A–D**). Gross views of the colon and cecum indicated severe colitis (**Figure 7E**). By contrast, both groups of mice that were administered either 12 mg/kg (LA-low) or 24 mg/kg (LA-high) of lauric acid displayed healthier colon and cecum morphology (**Figure 7E**), body weight recovery after infection (**Figure 7B**), a substantially lower body weight decrease (**Figure 7C**), and a higher cecum weight (**Figure 7D**) than the PBS control group. The protective effects of lauric acid appeared to be dose-dependent, as mice belonging to the LA-high group appeared to exhibit less severe symptoms of CDI compared with mice in the LA-low group. No differences were observed in the number of heat-resistant spores recovered from fecal samples (**Figure 7F**).

The expression levels of genes encoding for proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1βb), macrophage inflammatory

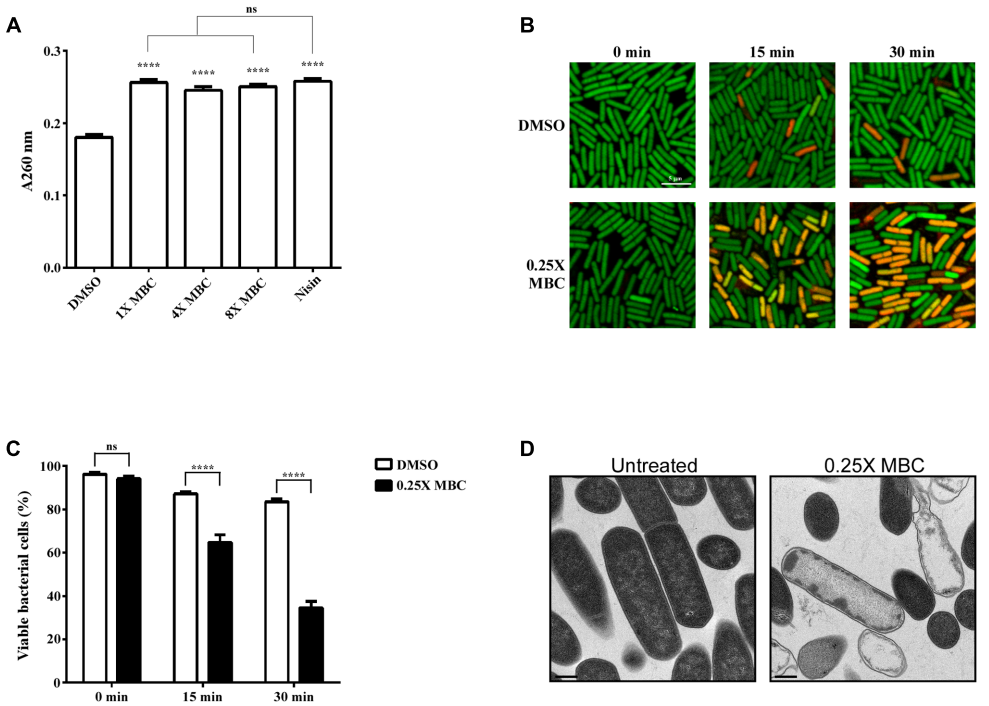

FIGURE 5 | Lauric acid induces bacterial cell membrane damage. To measure the damaging effect of lauric acid on *C. difficile* cell membrane, the vegetative cells of the strain R20291 were treated with various concentrations of lauric acid for up to 30 min, and cellular material leakage was quantified by measuring absorbance at 260 nm **(A)**. Nisin served as positive control. **(B)** Membrane permeability was measured by incubating cells with sublethal concentrations of lauric acid (0.25× MBC) for 15 and 30 min. Cells were then stained with SYTO9 (green) and propidium iodide (red) and imaged with confocal microscopy at 1,000× magnification. Scale bar = 5 μM. **(C)** Bacterial viability was quantified by counting the number of green fluorescent and red fluorescent cells from six images. **(D)** TEM analysis of vegetative cells treated with 0.25× MBC for 15 min compared with untreated control. Images were taken at 10,000× magnification, and scale bars = 0.5 μM. Results are the mean of three independent experiments. One-way ANOVA and two-way ANOVA was performed to assess significance for **(A)**, and **(C)**, respectively. ns, no significance; $^{****}P \leq 0.0001$.

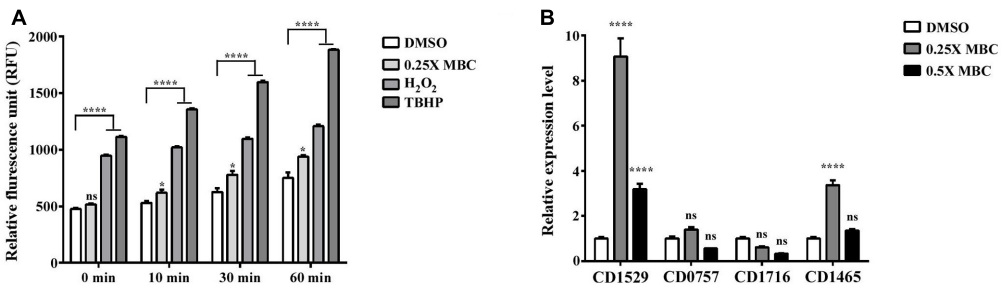

FIGURE 6 | Effect of lauric acid on intracellular ROS production and ROS-related genes in *C. difficile*. **(A)** *C. difficile* cells were treated with lauric acid for up to 60 min, and intracellular ROS was determined by staining with the general ROS indicator CM-H2DCFDA. H2O2 and TBHP served as ROS induction control. 1% DMSO – negative control. **(B)** The effect of lauric acid on ROS-related genes in *C. difficile*. *C. difficile* cells were treated with lauric acid or 1% DMSO for 30 min, and gene expression was then measured using real-time polymerase chain reaction. CDR20291_1529 (putative superoxide dismutase), CDR20291_0757 (putative superoxide reductase), CDR20291_1716 (putative peroxidase), and CDR20291_1465 (putative catalase). All data are presented as mean ± standard deviations, and statistical comparisons among groups were made using one-away ANOVA ($^{*}p \leq 0.05$, $^{****}p \leq 0.0001$). ns, not significant. All data are representative of at least three independent experiments.

protein 2 (MIP-2), and monocyte chemoattractant protein 1 (MCP-1) were considerably decreased in the colon homogenates of the LA-low and LA-high groups compared with those in the PBS group (**Figure 8A**). In addition to gene expression, proinflammatory cytokines and chemokines from the GAL fluid of all three groups were analyzed. Levels of TNF-α, IL-1β, and MCP-1 were significantly decreased in the GAL lavage fluid of both lauric acid treatment groups compared with that in the PBS control group ($P = 0.0254$, $P = 0.036$, and $P = 0.0285$, respectively, compared with the PBS control group) (**Figure 8B**).

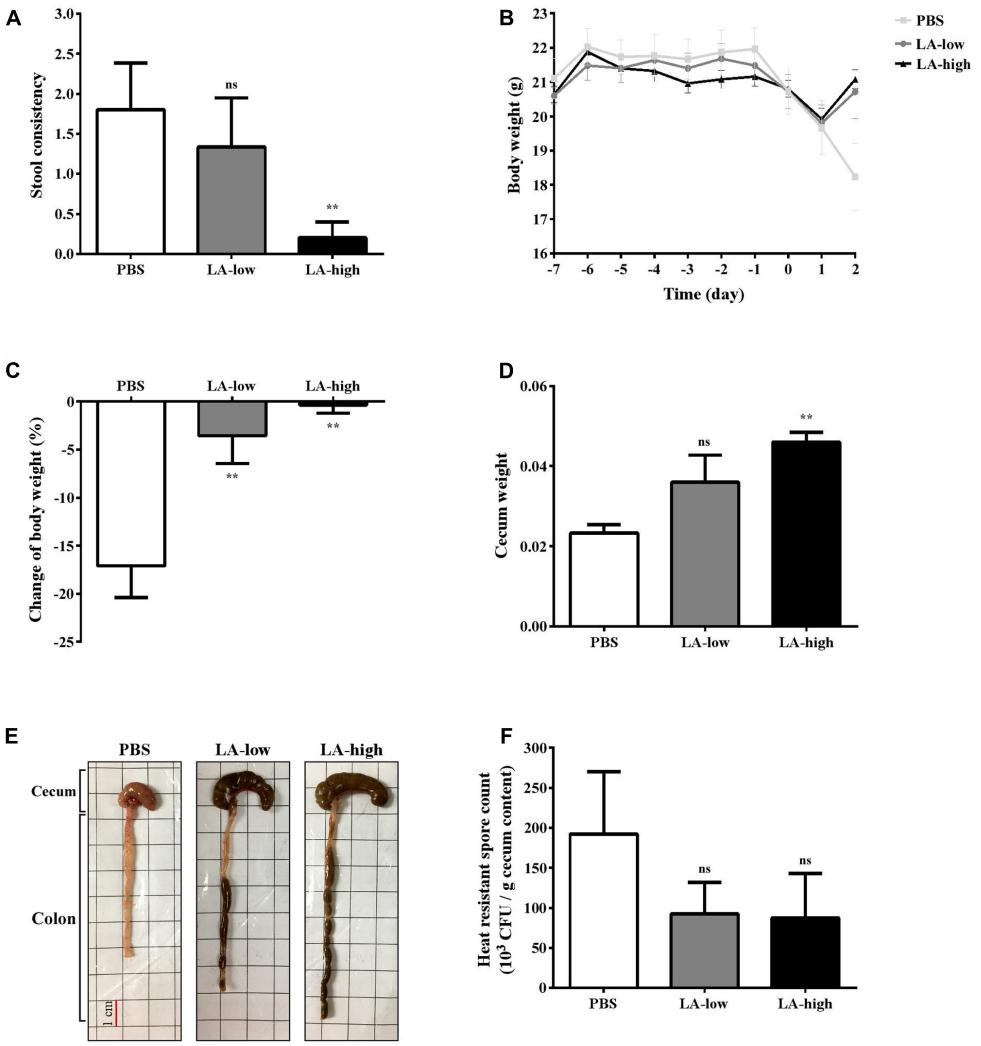

FIGURE 7 | Lauric acid treatment protects mice from *C. difficile* infection. Various groups of mice receiving lauric acid or PBS only were treated with an antibiotic cocktail and then challenged by *C. difficile* for 2 days. Stool consistency **(A)**, body weight over time **(B)**, body weight change **(C)**, cecum weight **(D)**, gross views of colon and cecum **(E)**, and heat-resistant fecal spore count 2 days post infection **(F)** were assessed. PBS: mice receiving PBS pre-treatment only; LA-low: mice receiving 12 mg/kg lauric acid; LA-high: mice receiving 24 mg/kg lauric acid. All data are presented as mean ± standard deviations, and statistical comparisons among groups were made using one-way ANOVA ($n = 5$, $^{**}P \leq 0.01$). ns, not significant. All data are representative of at least three independent experiments.

No statistically significant differences were observed in the levels of IL-6 and MIP-2 detected, although the trend was similar to that observed above. Collectively, these results demonstrated that the administration of lauric acid could decrease the severity of *C. difficile*-induced inflammation *in vivo*.

DISCUSSION

The main three drug resistance strategies of *C. difficile* are drug inactivation, target modification, and efflux pump, which have led to the emergence of hypervirulent drug-resistant strains (Harnvoravongchai et al., 2017). Noticeably, in recent years, the ability of *C. difficile* to tolerate multiple commonly prescribed antibiotics, its production of potent cytotoxins (toxin A, toxin B, and binary toxin CdtAB), and its high recurrence rate have resulted in CDIs becoming a healthcare concern worldwide (Martin et al., 2016). The current guideline for CDI treatment has focused on discontinuing previous antibiotic usage, and switching the treatment to metronidazole and vancomycin (Ananthakrishnan, 2011). However, similar to various other bacterial pathogens, *C. difficile* can develop antibiotic resistance; therefore, alternative treatment or prevention strategies are required. The development of new antibiotics, such as fidaxomicin, and monoclonal antibodies, such as bezlotoxumab, has provided clinicians with additional treatment options (Miller, 2010; Wilcox et al., 2017), but the prescription costs of these new medications remain high; thus, these drugs might not be readily accessible to economically disadvantaged patients. Furthermore, most new antibiotics are derivatives of existing

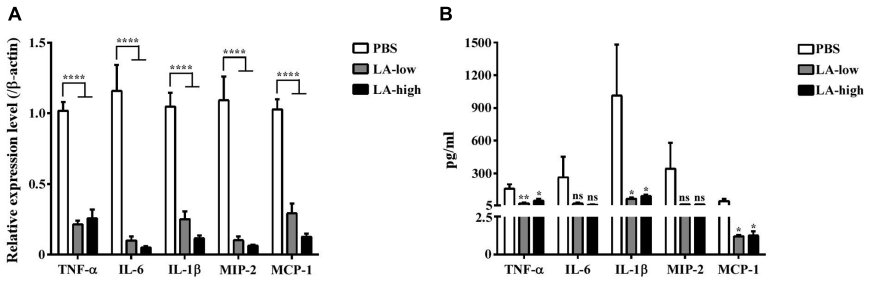

FIGURE 8 | Decreased level of proinflammatory cytokines in *C. difficile*-infected mice receiving lauric acid. The level of various proinflammatory cytokines and chemokines in colon tissues **(A)** and GAL **(B)** from mice belonging to the PBS treatment group, LA-low (12 mg/kg) group, and LA-high (24 mg/kg) group was measured using real-time polymerase chain reactions and ELISA, respectively. All data are presented as mean ± standard deviations, and statistical comparisons among groups were made using Student's *t*-test (*$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$). ns, not significant. All data are representative of at least three independent experiments.

ones and therefore share a similar mechanism of action and risk of drug resistance. The development of novel antibacterial products that are less likely to result in drug resistance in bacteria is therefore necessary. In the current study, we evaluated the inhibitory effects of various FAs on the growth of the *C. difficile* strain R20291. In our study, MCFAs, in general, were more effective in inhibiting *C. difficile* growth than SCFAs and LCFAs, with lauric acid exhibiting the lowest MBC. In contrast to the reported effect of SCFAs on bacterial pathogens, we revealed that SCFAs did not display considerable antibacterial activity against *C. difficile*. *C. difficile*, similar to many other members of the *Clostridium* genus, is known to produce various SCFAs such as butyric acid; therefore, *C. difficile* might have developed resistance mechanisms against these FAs (May et al., 1994; Ferreyra et al., 2014; Pettit et al., 2014).

Lauric acid is the major component of coconut oil, an edible oil extracted from the meat of coconuts. Lauric acid accounts for 45–53% of the overall FA composition of coconut oil; therefore, coconut oil is a dietary supplement that can modulate serum cholesterol levels (Katan et al., 1994; German and Dillard, 2004). When lauric acid is ingested, it is released from its triglyceride form and can either enter the liver through a portal vein or can be reformed into new triglycerides and enter the lymphatic system (Dayrit, 2015). In the serum, lauric acid is known to oxidize rapidly; therefore, only a small amount enters the liver. Once inside the liver, lauric acid is metabolized into acetyl-CoA for energy production, and some reaction products can also be transformed into ketone bodies, which also aid in energy production. The perception that lauric acid has beneficial effects is derived from studies that have indicated that lauric acid consumption increased serum high density lipoprotein (HDL), which is known to decrease the risk of coronary heart diseases (de Roos et al., 2001; Ekanayaka et al., 2013; Eyres et al., 2016). In addition to their ability to modulate cholesterol levels, the antimicrobial activity of MCFAs has been established for many years. Kabara et al. (1972) reported that compared with the other MCFAs screened (C6–C18), lauric acid showed the most potent effects *in vitro* against Gram-positive bacteria (Lynch et al., 1983; Carpo et al., 2007). Furthermore, the 1-monoglyceride form of lauric acid, monolaurin, exhibited an even higher potency, although its antibacterial range was reduced. Lauricidin^{TM}, which is composed of pure monolaurin, was patented as a nutritional supplement. Numerous studies have since been published on the antimicrobial activities of MCFAs against both Gram-negative and Gram-positive pathogens, with MBC values ranging from 0.068 to 0.375 mg/mL (Ruzin and Novick, 2000; Bergsson et al., 2001; Hinton and Ingram, 2006; Kitahara et al., 2006; Carpo et al., 2007; Nakatsuji et al., 2009; Fischer et al., 2012; Theinsathid et al., 2012). Shilling et al. (2013) reported that coconut-derived lauric acid, capric acid, and caprylic acid could inhibit the growth of *C. difficile in vitro*, whereas predigested virgin coconut oil exhibited a similar effect, although to a lesser degree. Shilling et al. (2013) also reported that lauric acid at 250 μM (MIC) could reduce bacterial growth by 90%. Although these previous studies have demonstrated the inhibitory effects of lauric acid in its pure form or as a derivative of lipolyzed virgin coconut oil, a research gap exits; none of these studies have revealed lauric acid's mode of action, its effect on CD physiology, and its effects on CDIs. In our study, we extended the antibacterial activity of lauric acid to multiple clinical isolates that included both toxigenic and non-toxigenic strains, and we revealed that the MBC ranged from 0.312 to 0.625 mg/mL, which supports the results of previous studies. We noted that the IC_{50} values for strain 630 and R20291 were considerably different, even though the MBC values for both strains were the same. Several possible reasons can be provided for this discrepancy. We used a more conservative approach for determining MBC; that is, we used the concentration at which no any growth was observed on agar plates. In addition, it has been reported that the MIC values for two bacterial strains were the same, whereas their IC_{50} values were considerably different (Barbour et al., 2016). In addition to the inhibitory effect observed on vegetative cells, lauric acid could inhibit biofilm formation. At 0.25× MBC, lauric acid was equally as effective at reducing biofilm formation as vancomycin applied at 20 μg/mL. This effect was probably due to the inhibition of all cell growth. More interestingly, lauric acid disrupted preformed biofilms, and this biofilm-damaging effect had not been reported in other studies. Dapa et al. (2013) reported that R20291 *in vitro* forms biofilms with higher mass than strain 630 does, which corroborates our observation that the

preformed biofilm of the strain 630 was significantly disrupted by 1× MBC of lauric acid, in contrast with the biofilm of the strain R20291, which required 2× MBC of lauric acid for a disruptive effect. Future studies should investigate the effect of lauric acid on biofilm reduction by comparing the presence of live or dead cells in the retained biofilm. At present, the exact mechanism underlying biofilm removal by lauric acid is unclear. It is possible that the mild detergent effect of lauric acid not only damages cell membranes, but also bacterial adhesins that contribute to biofilm formation. Importantly, the biofilm removal effect of lauric acid supports its potential use as an antibacterial agent.

Previous studies have indicated that MCFAs, including lauric acid, can inhibit the outgrowth of *Bacillus* and *Clostridium* spores, although *C. difficile* was not included in these studies (Ababouch et al., 1992; Shearer et al., 2000). In our study, we also observed that lauric acid treatment was effective at reducing spore outgrowth. Spore germination is a dynamic process that is initiated by the hydration of the spore cortex, followed by the release of DPA, which can be monitored by changes in OD and by measuring the DPA level in the supernatant. In the present study, the rate of spore germination was increased in the presence of lauric acid, but no differences were observed in the rate of DPA release. We hypothesize that lauric acid accelerates the rate of germination in the presence of the germinant TA, and that this hastens the killing of the eventually germinated vegetative cells. Studies are currently underway to address this possibility. Additional studies are required to determine whether lauric acid or other MCFAs can disrupt bacterial spore coats to enhance spore germination.

In the present study, we observed a significant increase in membrane permeability and the release of cytoplasmic materials, consistent with the membrane-damaging effect of lauric acid reported in previous studies (Nobmann et al., 2010). Currently, it is unclear how lauric acid penetrates the cell wall to reach membrane sites. We reported almost equal nucleic acid material release rates among nisin (positive control) and all lauric acid treatment groups (1×–8× MBC). Furthermore, lauric acid significantly induced ROS generation and significantly increased the expression of genes potentially associated with oxidative damage defense. The lower expression level of these genes in cells treated with a high concentration of lauric acid than in cells treated with a lower concentration of lauric acid might be due to the rapid toxicity of lauric acid, which suppressed bacterial metabolism. Recently, Kint et al. (2017) reported that the alternative sigma factor σ^B is involved in protection against ROS. Interestingly, although no significant differences were observed in the expression level of CDR20291_0757 and CDR_1716 in this study, their homologs in the strain 630 were differentially regulated by σ^B. ROS regulation may be different between the two strains, or that ROS generation induced by lauric acid in our study might induce other genes. Future studies should conduct a transcriptomic analysis to increase our understanding of the extent to which lauric acid treatment affects gene expression in *C. difficile*.

In our *in vivo* experiments, we observed that daily lauric acid intake significantly reduced the severity of diarrhea and intestinal inflammation associated with CDI. It is still unclear whether the direct killing effect of lauric acid on *C. difficile* observed *in vitro* was involved in the reduction in the inflammation observed *in vivo*. Additional animal studies should focus on increasing the sample size, a longer postinfection observation, and determining the luminal lauric acid concentration during infection. If the concentration of luminal lauric acid reaches a similar level as the MIC determined in this study, then the reduction in inflammation might be due to the direct killing of *C. difficile* in the gut. It is also possible that the luminal lauric acid concentration did not reach the MIC, which suggests that lauric acid acted upon the host to reduce inflammation. In this study, the observation that the number of fecal spores between lauric acid-treated and control groups was similar indicated an indirect effect of lauric acid *in vivo*. It has been reported that MCFAs, including lauric acid, are partial PPAR-α and PPAR-γ agonists, which are known to exert anti-inflammatory effects (Kliewer et al., 1997; Clark, 2002; Croasdell et al., 2015). However, more experiments are required to clarify these outstanding questions. Collectively, the results of this study indicate that lauric acid exhibits potent antibacterial activity against *C. difficile*, and lauric acid prophylaxis may substantially decrease the level of inflammation induced by infection with *C. difficile in vivo*. The beneficial effect of lauric acid as a food supplement or as an adjunct therapy for CDI should be considered.

AUTHOR CONTRIBUTIONS

I-HH, H-TY, JR, and J-WC designed the experiments. H-TY, Y-ZJ, and J-WC carried out the experiments. I-HH, H-TY, JR, J-WC, P-JT, YP-H, DP-S, and W-CK analyzed the data. H-TY, J-WC, and I-HH prepared the manuscript.

FUNDING

This study was funded by the Taiwan Ministry of Science and Technology grant number MOST 106-2321-B-006 -008 to I-HH, grant number MOST-103-2320-B-006-028-MY2 to J-WC and from the Comisión Nacional de Ciencia y Tecnología de Chile (FONDECYT Grant 1151025) to DP-S. This manuscript was edited by Wallace Academic Editing.

ACKNOWLEDGMENTS

We wish to thank the members of the IHH lab and laboratory personnel of P-JT for providing technical services. We also wish to thank Dr. Shang-Rung Wu for her assistance with TEM analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.02635/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CS and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 March 2018
doi: 10.3389/fmicb.2018.00415

Mutacin 1140 Lantibiotic Variants Are Efficacious Against *Clostridium difficile* Infection

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Edited by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 08 November 2017
Accepted: 21 February 2018
Published: 16 March 2018

Citation:
Kers JA, Sharp RE, Defusco AW, Park JH, Xu J, Pulse ME, Weiss WJ and Handfield M (2018) Mutacin 1140 Lantibiotic Variants Are Efficacious Against Clostridium difficile Infection. Front. Microbiol. 9:415. doi: 10.3389/fmicb.2018.00415

Lantibiotics offer an untapped pipeline for the development of novel antibiotics to treat serious Gram-positive (+) infections including *Clostridium difficile*. Mutacin 1140 (MU1140) is a lantibiotic produced by *Streptococcus mutans* and acts via a novel mechanism of action, which may limit the development of resistance. This study sought to identify a lead compound for the treatment of *C. difficile* associated diarrhea (CDAD). Compounds were selected from a saturation mutagenesis library of 418 single amino acid variants of MU1140. Compounds were produced by small scale fermentation, purified, characterized and then subjected to a panel of assays aimed at identifying the best performers. The screening assays included: *in vitro* susceptibility testing [MIC against *Micrococcus luteus*, *Clostridium difficile*, vancomycin-resistant enterococci (VRE), *Staphylococcus aureus*, *Streptococcus pneumonia*, *Mycobacterium phlei,* and *Pseudomonas aeruginosa*; cytotoxicity screening on HepG2 hepatocytes; *in vitro* pharmacological profiling with the Safety Screen 44^{TM}, metabolic and chemical stability in biologically relevant fluids (FaSSGF, FaSSIF and serum); and efficacy *in vivo*]. Several lantibiotic compounds had better MIC against *C. difficile*, compared to vancomycin, but not against other bacterial species tested. The Safety Screen 44^{TM} *in vitro* pharmacological profiling assay suggested that this class of compounds has relatively low overall toxicity and that compound OG253 (MU1140, Phe1Ile) is not likely to present inadvertent off-target effects, as evidenced by a low promiscuity score. The *in vitro* cytotoxicity assay also indicated that this class of compounds was characterized by low toxicity; the EC_{50} of OG253 was 636 mg/mL on HepG2 cells. The half-life in simulated gastric fluid was >240 min. for all compound tested. The stability in simulated intestinal fluid ranged between a half-life of 5 min to >240 min, and paralleled the half-life in serum. OG253 ultimately emerged as the lead compound based on superior *in vivo* efficacy along with an apparent lack of relapse in a hamster

model of infection. The lessons learned from this report are applicable to therapeutic lanthipeptides in general and may assist in the design of novel molecules with improved pharmacological, therapeutic and physicochemical profiles. The data presented also support the continued clinical development of OG253 as a novel antibiotic against CDAD that could prevent recurrence of the infection.

Keywords: lanthipeptide, antibiotic, bacteriocin, nisin, mutagenesis, structural variant, resistance

INTRODUCTION

The Centers for Disease Control (CDC) report on Antibiotic Resistance Threats in the United States (Centers for Disease Control and Prevention [CDC], 2013) classifies *Clostridium difficile* infection as an urgent threat, based on the high incidence of 250,000 infections per year and a mortality rate of 14,000 deaths per year. Due to increasing rates of mortality and the emergence of strains with increased virulence, it is anticipated that the current cost of medical care, which currently exceeds $1 billion per year in the US alone, will continue to increase. Although the epidemiology of *C. difficile* infection (CDI) can vary markedly in different countries, with many experiencing high rates of CDI and general incidence rates that are increasing (Wilcox, 2016). This crisis has ushered in national programs aimed at increasing antibiotic stewardship and recently added new drugs/biologicals in the pharmacopeia. In 2011, the FDA/EMA approved fidaxomicin (Dificid) (Vaishnavi, 2015), while the antitoxin antibody bezlotoxumab (Zinplava) was recently approved by the FDA and the EMA in 2016 and 2017, respectively (Kufel et al., 2017). While new options for infectious disease treatment have been welcomed, there is concern that their high cost may prevent wide-spread use (Surawicz et al., 2013). Nevertheless, there continues to be extensive research efforts due to the high unmet clinical need to develop additional novel strategies to combat *C. difficile* infections, as presented in this special Research Topic Issue, including novel small molecule and peptide-derived antimicrobials, and extending to alternative approaches such as fecal transplantation (FMT), microbial restoration and phage therapy, *etc*.

Lantibiotics are a relatively old class of compounds that were initially discovered around the same time as penicillin; the archetype lantibiotic nisin was first discovered in 1928 and subsequently isolated in 1947 (Rogers and Whittier, 1928; Field et al., 2015). Lantibiotics are part of a larger class of lanthipeptides that derive their name from the thioether ring containing amino acids lanthionine (Lan, Ala-S-Ala) and/or 3-methyl-lanthionine (MeLan, Abu-S-Ala). These compounds often incorporate post-translationally modified amino acids such as Dha, Dhb, and the unsaturated lanthionine derivatives AviCys at their *C*-terminus (reviewed in Chatterjee et al., 2005; Smith and Hillman, 2008; Ross and Vederas, 2011; see **Figure 1** and **Table 1**).

Despite a substantial amount of evidence that demonstrate that lantibiotics are efficacious and that they would be well tolerated in humans (Smith and Hillman, 2008; Ross and Vederas, 2011; Piper et al., 2012; Götz et al., 2014; Field et al., 2015; Ongey et al., 2017), only a few have advanced to clinical trials: NVB333 (methicillin-resistant *Staphylococcus aureus* infections (MRSA) and VRE infections) are currently in pre-clinical testing, NVB302 (*C. difficile* infections) completed Phase-I, and Duramycin (Cystic Fibrosis) completed Phase-II (Sandiford, 2015; Boakes et al., 2016; Ongey et al., 2017).

Mutacin 1140 is a lantibiotic produced by the Gram-positive bacterium *Streptococcus mutans* that was discovered independently by two different groups (Hillman et al., 1984, 1998; Qi et al., 1999). Thirty-three years were spent characterizing, producing and purifying enough of the compound to sufficient quantities with high enough purity to investigate its potential as a novel therapeutic agent. During that period, the polycistronic operon of MU1140 was mapped (Hillman et al., 1998) and entirely sequenced (Escano et al., 2016), its *in vitro* susceptibility profile and spectrum of activity were established (Ghobrial et al., 2009), a low frequency of development of antimicrobial resistance was substantiated (Ghobrial et al., 2009), the low levels of cytotoxicity and good overall pharmacological profile were reported (Ghobrial et al., 2009, 2010; Oragenics, unpublished), and its high-resolution structure defined by nuclear magnetic resonance (NMR) spectroscopy (Smith et al., 2003). Of particular interest, MU1140 was found to exert its antimicrobial activity via a novel mechanism of action termed Lipid-II abduction (Hasper et al., 2006; de Kruijff et al., 2008; Smith et al., 2008), which is less prone to natural development of resistance because of the ancestral nature of the pyrophosphate moiety of Lipid-II (Ghobrial et al., 2009). Lipid II is an essential component of peptidoglycan synthesis that is targeted by several antibiotics, including vancomycin (Brötz et al., 1998). In contrast to vancomycin, which binds to Lipid II at the terminal part of the pentapeptide, MU1140 (and nisin) binds the pyrophosphate moiety of Lipid-II. Following selection by exposure to vancomycin, bacterial species have evolved the ability to change the composition of the pentapeptide of Lipid-II without impeding cell wall synthesis. For instance, VREs that acquire *van*A develop vancomycin-resistance by the substitution of the *D*-Ala-*D*-Ala of the pentapeptide of Lipid-II by a *D*-Ala-*D*-Lactate moiety (Hsu et al., 2004). In contrast, no bacterial species have yet been identified that can effectively modulate the structure of the pyrophosphate moiety of Lipid-II without impacting its function, which makes it an attractive target for the development of antibiotics. Consequently, the critical and ancestral nature of the pyrophosphate moiety of Lipid-II implies that the development of resistance would be infrequent (Ghobrial

Abbreviations: Abu, aminobutyric acid; AviCys, aminovinyl-*D*-cysteine; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; Lan, lanthionine; MeLan, methyl-lanthionine; MU1140, Mutacin 1140.

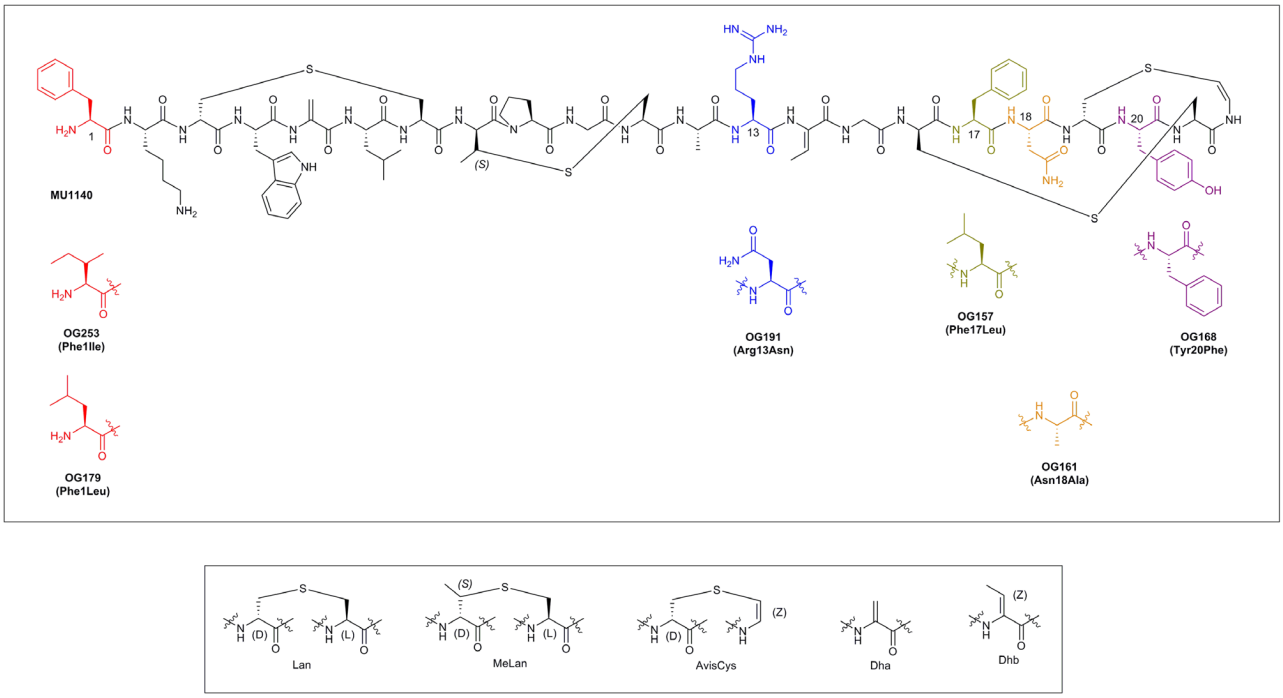

FIGURE 1 | The chemical structure of MU1140 and top 6 variants (top). Substitutions are displayed in parenthesis. Post-translationally modified residues in lantibiotics are presented in the (bottom).

et al., 2009). The use of nisin in the food industry since 1951 (in over 50 countries) certainly supports that hypothesis.

Mutacin 1140 (see **Figure 1**) is an attractive scaffold for future antibiotic engineering by virtue of its excellent safety profile, novel mechanism of action and consequently its limited potential for development of resistance in the clinic. We have previously proposed a new set of blueprints that may enable and foster future therapeutic development of lantibiotics, taking into account classic pitfalls related to their production, purification and formulation. These issues have historically hindered the "drugability" of MU1140 as well as other lantibiotics. A saturation mutagenesis library was previously created to study the contribution of every amino acids of the core peptide of MU1140, in an unbiased and addressable fashion (Kers et al., unpublished), to create 418 different variants of MU1140 (22 positions × 19 possible permutations). In the current report, we triage the best performers from the saturation mutagenesis library in order to select a lead compound for the treatment of *C. difficile* infections based on: (a) physicochemical properties, (b) *in vitro* susceptibility levels, (c) spectrum of activity, (d) stability and solubility in bio-relevant fluids (FaSSGF, FaSSIF and serum), and (e) efficacy *in vivo*.

MATERIALS AND METHODS

Strains

The construction of the MU1140 variant strain library has been detailed previously (Kers et al., unpublished) see **Table 2**. Briefly, variants were constructed in *S. mutans* JH1140 (Hillman et al., 1984) by allelic replacement where the native chromosomal *lan*A gene was replaced with *lan*A variants encoding codon substitutions. Splicing by Overlap Extension (SOE) PCR was used to construct DNA vectors for integration of *lan*A variants into the JH1140 chromosome using a selectable erythromycin resistance marker (from pVA891, Macrina et al., 1983). Strains were routinely grown on triptic soy broth or agar containing 0.2% yeast extract (TSYEX), 3 μg/mL erythromycin, and incubated in a candle jar for 3 days at 37°C. PCR and Sanger DNA sequencing was utilized to confirm replacement of the chromosomal copy of *lan*A with

TABLE 1 | Amino-acid sequence of the core peptide precursor and post-translationally modified MU1140.

Precursor	Ser3	Ser5	Cys7	Thr8	Cys11	Thr14	Ser16	Ser19	Cys21	Cys22
Modified	**Ala3**	Dha5	**Ala7**	**Abu8**	**Ala11**	Dhb14	**Ala16**	**Ala19**	**Ala21**	**Avi-Cys22**

**Only post-translationally modified residues are displayed. Bolded residues are involved in a lanthionine (Lan, Ala-S-Ala) and/or 3-methyl-lanthionine (MeLan, Abu-S-Ala) bridges. Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; AviCys, aminovinyl-D-cysteine.*

TABLE 2 | Selected[1] strains used in this study.

Name	Property, genotype or characteristics	Source or reference
S. mutans JH1140	MU1140 hyperproducing strain of *S. mutans* derived from JH1000	Hillman et al., 1984
S. mutans SM152	JH1140::Erm (intergenic *lan*A'-*lan*B) (producing MU1140)	Kers et al., unpublished
S. mutans SM126	*S. mutans* JH1000 ΔlanAA′ (*lan*A$^-$)	Kers et al. unpublished
S. mutans SM157	SM152 producing OG157 (Phe17Leu)	Kers et al., unpublished
S. mutans SM161	SM152 producing OG161 (Asn18Ala)	Kers et al., unpublished
S. mutans SM168	SM152 producing OG168 (Tyr20Phe)	Kers et al., unpublished
S. mutans SM179	SM152 producing OG179 (Phe1Leu)	Kers et al., unpublished
S. mutans SM191	SM152 producing OG191 (Arg13Asn)	Kers et al., unpublished
S. mutans SM253	SM152 producing OG253 (Phe1Ile)	Kers et al., unpublished
S. aureus ATCC 29213	CLSI control, methicillin susceptible	Eurofins
S. aureus ATCC 19636	Smith, Osteomyelitis isolate, *in vivo* strain	Eurofins
S. aureus ATCC 33591	MRSA, *in vivo* strain	Eurofins
S. aureus BAA-1717	Community acquired MRSA USA300 SCCmec type IVa PVL+	Eurofins
S. aureus ATCC 700699	Mu50 VISA	Eurofins
S. pneumonia ATCC 700675	PEN-R Macrolide-R, South Africa 6B-8	Eurofins
M. phlei ATCC 11758	–	Eurofins
P. aeruginosa TUH-974	MDR clinical isolate	Eurofins
C. difficile ATCC 9689	Ribotype 001	Eurofins
C. difficile BAA-1805	Ribotype 027 (NAP1) hypervirulent strain	Eurofins
C. difficile BAA-1875	Ribotype 078	Eurofins
C. difficile UNT103-1	VA11, non epidemic (cdtB-, REA group J)	UNTHSC[2]
E. faecalis ATCC 51575	VRE VanB strain	Eurofins
E. faecalis ATCC 51299	VRE VanB strain	Eurofins
E. faecium ATCC 700221	VRE VanA strain	Eurofins

[1]*Entire S. mutans strains SMxxx library can be found in Kers et al., (unpublished).* [2]*Received from Ohio VA Medical Center (Curtis Donskey).*

the *lan*A variant encoded on the integration vector. A high-throughput method was used to confirm the biological activity of each variant strain using *Micrococcus luteus* as a reporter strain (see details in Kers et al., unpublished). Strains are denoted as SMxxx, while their corresponding lantibiotics are labeled as OGxxx (e.g., strain SM253 produces the lantibiotic OG253).

Compound Manufacturing and Characterization by Ultra-High Performance Liquid Chromatography/Mass Spectrometry (UPLC/MS), HPLC and Nuclear Magnetic Resonance

Compounds tested in this study were produced by fermentation and purified by Intrexon Corp. Detailed experimental conditions have been previously reported (Kers et al., unpublished). Larger scale (∼100 mg) manufacturing was carried out at Oragenics using proprietary methods (Oragenics, unpublished). Briefly, MU1140 chromosomal *lan*A variant strains were grown in Sartorius Biostat A plus 1 L bioreactor using fed-batch fermentations under aerobic stirred-tank conditions with automated temperature/pH/dissolved oxygen controls. Culture supernatants were extracted using chloroform extractions to isolate the compounds of interest (modified from Hillman et al., 1984). Purification was carried out by flash chromatography. This scale was adequate to generate sufficient purity and quantities of compounds to perform all assays contained in the current report.

The purity and identity of each variant was determined by UPLC/MS as previously reported (Kers et al., unpublished). The purity of variants during solubility and stability studies was determined by HPLC on a Waters XBridge C18 column, particle size 3.5 μm, 3.0× 150 mm. Buffer A was 0.05% trifluoroacetic acid (TFA) in H_2O and Buffer B was 0.05% TFA in 5% H_2O/95% acetonitrile (ACN). The gradient was 2% buffer B to 85% buffer B over 38 min at flow rate of 0.6 mL/min. Injection volume was 100 μL.

NMR spectra of OG253 (MU1140-Phe1Ile) were acquired on an Agilent NMRS instrument, operating at 600 MHz for proton, and equipped with a High Temperature Superconductor (HTS) 1.5 mm probe (University of Florida NMR Core Facility). The water signal was suppressed in a wet 1D experiment. The precedent parameters were used to setup: (a) a Total Correlation Spectroscopy (TOCSY) experiment with a mixing time of 150 ms, (b) a Rotating-Frame NOE Spectroscopy (ROESY) experiment with a mixing time of 200 ms, (c) a Heteronuclear Single Quantum Coherence (HSQC) experiment optimized for a one-bond coupling constant of 146 Hz and (d) a Heteronuclear Multiple Bond Correlation (HMBC) experiment optimized for a long-range coupling constant of 8 Hz. A 5 mg sample of OG253 was dissolved in 60 μL of a mixture of deuterated acetonitrile:water, 3:1, yielding a 37 mM solution (Smith et al., 2000).

Minimum Inhibitory Concentration (MIC) Testing

MIC testing was performed by Eurofins Panlabs Taiwan LTD. Briefly, MICs were measured using the broth dilution assay as described by the CLSI for aerobic bacteria (CLSI, 2009), and a modification of the CLSI M11-A8 broth dilution method for *Bacteroides fragilis* susceptibility testing was used for *C. difficile* (CLSI, 2012). The strains tested are presented in **Table 2**. Briefly, lyophilized compounds were dissolved and diluted with 100% dimethyl sulfoxide (DMSO). All compounds were tested with a standard reference agent for each strain (ampicillin, gentamicin, vancomycin, ofloxacin, or linezolid). Each test article or reference agent were tested at 11 concentrations by twofold serial dilutions from 64 to 0.0625 μg/mL, incubated and then visually examined and scored positive (+) for inhibition of growth or turbidity or negative (−) for no effect upon growth or turbidity. The final concentration of DMSO was 2%. Each concentration was evaluated in duplicate. The MIC was defined as the lowest concentration of an antimicrobial agent that completely prevented visible growth of a microorganism in the broth susceptibility test. Vehicle-control and active reference agents were used as blank and positive controls, respectively. The MIC values of ampicillin, gentamicin, vancomycin and ofloxacin controls were consistent with the historical data against test strains (data not shown).

In Vitro Cytotoxicity

The HepG2 cytotoxicity evaluation was performed by MB Research Labs (Spinnerstown, PA, United States) by the Neutral Red Uptake Bioassay of HepG2 human hepatocellular carcinoma cells in culture (ATCC #HB-8065). Briefly, HepG2 cells were seeded in the central 60 wells of 96-well plates and maintained in culture for approximately 24 h. Following pre-incubation, the culture medium was replaced with culture medium containing increasing concentrations of the test articles or control in the vehicle. Test articles were tested in triplicate wells, and reported as the mean. The cells were incubated for 24 h and cell viability was determined by Neutral Red (NR) uptake. For this assay, the medium was then replaced with NR medium (50 μg/mL NR in Hanks' Balance Salt Solution, HBSS) and the cells were incubated for an additional 3 h. Following uptake of NR, the medium was discarded, the wells were washed with HBSS, and lysing solution (50:49:1 ethanol:water:acetic acid) was added subsequently to each well. The plates were placed on a rocker for 30 min until NR had been extracted from the cells and formed a homogeneous solution. The absorbance of each well was measured at 540 nm on a μQuant™ plate reader (Bio-Tek Instruments) using KCjunior™ software. The mean of the outer wells (DMEM2-FBS) was used as a reference control. The mean percent viability was determined for each concentration compared to the vehicle-treated control. A positive result was determined by: (1) a >25% decrease in absorbance of test article-treated cells calculated as a percentage of vehicle-treated cells, and/or (2) $p < 0.05$ of test article-treated cells using the Student's *t*-test. Where possible, the concentration at which cell viability was reduced to 50% (EC_{50}) was calculated.

In Vitro Safety Screen 44™ Pharmacologic Profiling

The *in vitro* pharmacology profiling was performed by CEREP Labs (now Eurofins Panlabs, Celle-Lévescault, France). Compounds were tested in Safety Screen 44™ panel, which is for off-target profiling using a compilation of classical competition binding assays and enzymatic inhibition assays performed with human recombinant proteins (Bowes et al., 2012). Experimental parameters and conditions for each of these 44 assays were as per the manufacturer's testing information[1]. The data reported is expressed as the mean of duplicate experiments. The respective reference compound was tested concurrently with the test compounds, and the data were compared with historical values determined at Cerep. The experiment was accepted in accordance with Cerep's validation Standard Operating Procedure. The Safety Screen 44™ results showing an inhibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effects of the test compounds. 50% is the most common cut-off value for further investigation (determination of IC_{50} or EC_{50} values from concentration-response curves) that the manufacturer recommends, based on historical data. Results showing an inhibition (or stimulation) between 25 and 50% are indicative of weak to moderate effects (in most assays, they should be confirmed by further testing as they are within a range where more inter-experimental variability can occur). Results showing an inhibition (or stimulation) lower than 25% are considered insignificant and mostly attributable to variability of the signal around the control level. High negative values (≥50%) that are sometimes obtained with high concentrations of test compounds are generally attributable to non-specific effects of the test compounds in the assays. On rare occasions they could suggest an allosteric effect of the test compound.

Metabolic Stability in Fasted State Simulated Gastric Fluid (FaSSGF), Fasted State Simulated Intestinal Fluids (FaSSIF) and Serum

The studies highlighted below were performed by Seventh Wave Laboratories and the University of Massachusetts, Lowell (see **Table 3**). Fasted State Simulated Gastric Fluid (FaSSGF) was prepared with 2.0 g NaCl in 7.0 mL conc. HCl and sufficient water (QS) to make 1000 mL (final pH 1.5), with and without 3.2 g purified porcine pepsin (800 – 2500 U/mg, Fisher Scientific). Pepsin is the major proteolytic enzyme produced in the stomach and it is known to cleave peptides with an aromatic acid on either side of the peptide bond (Fruton, 2002). A stock of each of the compounds for testing was prepared at 10 mg/mL in 10% ACN. Initially, 242.5 μL of FaSSGF ± pepsin was added to snap-lid 1.5 mL Eppendorf tubes. To each tube, 7.5 μL of compound at 10 mg/mL was added for a final concentration of 300 μg/mL. All tubes were incubated at 37°C. At each time point, a 40 μL aliquot was removed from each tube, and the protease activity

[1]Cerep Safety Screen 44: https://www.eurofinsdiscoveryservices.com/catalogmanagement/viewitem/SafetyScreen44-Panel-Cerep/P270

TABLE 3 | Metabolic and chemical stability, solubility and half life in biologically relevant fluids.

Compound[1]	30% HPβCD		5% Mannitol		Half-life		
	Solubility[2] (mg/ml)	Stability[2,3] (%)	Solubility[4] (mg/ml)	Stability[3,4] (%)	FaSSGF[4] (min)	FaSSIF[5] (min)	Serum[5] (min)
MU1140	<8	−7.6	41.0	−0.2	>240	72.4	107
OG157 (Phe17Leu)	<8	−3.2	166.5	0.4	ND	4.9	37.1
OG161 (Asn18Ala)	<8	0.3	181.6	−0.1	>240	<30	<60
OG168 (Tyr20Phe)	<8	−0.4	41.5	−0.3	>240	148	195
OG179 (Phe1Leu)	<8	−2.0	161.6	0.4	>240	77.3	60.8
OG191 (Arg13Asn)	29	6.0	39.7	−0.9	>240	>240	>107
OG253 (Phe1Ile)	<8	−13.4	169.2	0.2	>240[6]	95.0	270

[1]Substitutions from MU1140 in parenthesis. [2]Performed at Almac Group. [3]Stability expressed as the difference of purity pre- versus post-incubation. Incubation at room temperature, 72 h. [4]Performed at UMASS Lowell. Data reported as the mean of triplicate experiments. [5]Performed at Seventh Wave Laboratory, with confirmations at UMASS Lowell. Data reported as the mean of triplicate experiments. [6]Confirmed at Oragenics.

in the reaction aliquot was inactivated by addition of 1 μL of formic acid and 120 μL of ACN. Time points collected were at 0, 0.5, 1, 2, and 4 h. For negative control, only 0 and 4 h time points were collected. Post-precipitated samples were centrifuged at 20,000 × *g* for 10 min in a table top centrifuge at room temperature. A 75 μL portion of the supernatant was transferred to a HPLC vial and 450 μL of 0.05% TFA in water was added for HPLC analysis (see HPLC Method above).

Fasted State Simulated Intestinal Fluid (FaSSIF) was prepared as follows: FaSSIF-v2[2] solution in maleate buffer which has the composition of 3 mM sodium taurocholate, 0.2 mM lecithin, 19.12 mM maleic acid, 34.8 mM NaOH, 68.62 mM NaCl (final pH 6.5), with and without protease (2% Pancreatin (w/v), 6 μg/mL of trypsin and 6 μg/mL of α-chymotrypsin (Foltmann, 1981). All proteases were from Fisher Scientific. A stock of each compound under investigation was prepared at 10 mg/mL in 10% ACN. Initially, 7.5 μL samples of compound at 10 mg/mL were added to 1.5 mL Eppendorf tubes, and 242.5 μL of FaSSIF ± enzymes were added to each sample (final concentration of compound of 300 μg/ml). Samples were incubated at 37°C in closed tubes. Time points collected were at 0, 0.5, 1, 2, and 4 h. For FaSSIF without enzymes (negative control), only 0 and 4 h time points were collected. At each time point, a 40 μL aliquot was taken from each tube. The protease activity in the aliquot was stopped and the proteins precipitated with 1 μL of formic acid and 120 μL of ACN. Precipitated samples were then centrifuge at 20,000 × *g* for 10 min in a table top centrifuge at room temperature. A 75 μL volume of supernatant was transferred to an HPLC vial and 450 μL of 0.05% TFA in water was added for HPLC analysis using the previously described method.

Stability in human serum was tested as follows: a 10 mg/mL solution of each compound was made in 10% ACN. A 7.5 μL volume of the 10 mg/mL compound stock was added to 242.5 μL of 100 mM acetate buffer at (pH 5.5) in a 1.5 mL Eppendorf tube (for a final concentration of). A 242.5 μL volume of complement preserved serum (0.2 μm filtered, pooled gender, Bioreclamation, Cat# HMSRM-COMP) was added to the tube (final concentration of 300 μg/mL per compound). Negative controls did not contain serum. To both the serum tube and the negative tube, samples were incubated covered at 37°C. Time points collected were 0, 0.5, 1, 2, 4, and 8 h. For negative control, only the time 0 and 8 h time points were collected. At each time point, a 40 μL aliquot was taken from each tube for HPLC analysis (see Materials and Methods).

All metabolic stability FaSSGF, FaSSIF and serum data is reported as the mean of triplicate experiments.

Efficacy Assessment *in Vivo*

Animals and Test Organism

Male Golden Syrian hamsters were used in this study weighing 80 – 90 g (Charles River Laboratories, Wilmington, MA, United States), housed one per cage with free access to food and water, in accordance with NIH guidelines. *C. difficile* UNT103-1 (VA11, non-epidemic (cdtB-, REA group J) was received from Curtis Donskey, Cleveland VA Hospital, Cleveland, OH, United States). The isolate has been previously utilized for the hamster model (Weiss et al., 2014) and is part of the University of North Texas Health Science Center culture collection and was preserved in a Trypic Soy Broth stock containing 20% glycerol at −70°C. This study was carried out in accordance with protocols 2012/13-14-A06, 2016-0019, and 2016-0015 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Texas Health Science Center (UNTHSC). IACUC established guidelines ensuring that approved protocols are in compliance with federal and state laws regarding animal care and use activity at UNTHSC. The UNTHSC animal program is USDA registered (74-R0081) and fully AAALAC accredited.

Surgical Procedure

On the day of surgery, each animal was anesthetized with 3–4% isoflurane and a midline incision generated through the ventral peritoneum. While retracting the incision, the ileum proximal to the ileocecal junction was isolated and temporarily ligated with 2 – 0 silk suture. A 1 – 2 mm transverse incision was made within the ligated ileal section and the tippet of an MRE-40 catheter (Braintree Scientific) inserted through the incision and advanced into the ileum to a length of ∼1 cm toward the ileocecal junction. The catheter was anchored to the ileum with 5 – 0 monofilament

[2]http://www.Biorelevant.com

suture, and the cannulated ileum placed back into the peritoneal cavity. The peritoneal incision was then closed and the remainder of the catheter routed subcutaneously (SC) for external porting through the skin near the scapular region. Animals were given 2 mg/Kg meloxicam as needed for pain and discomfort. Animals recovered for a period of 10 days after surgery before infection.

Surgical Days and Infection

A total of 60 animals were implanted with ileal cannulas on 3 – 4 separate days (to achieve 48 animals for study), as presented in **Table 4**. Animals from all days were randomized into the 8 study groups (N = 6 per group) prior to treatment. UNT103-1 was passaged onto trypticase soy agar supplemented with 5% sheep blood (TSA + SB) plates 4 days before infection and anaerobically incubated in an anaerobic chamber at 37°C for 48 h. After incubation, the plate growth was suspended into 20 mL of pre-reduced tryptone-glucose-yeast extract (TGY) nutrient broth and anaerobically incubated at 37°C for 24 h. At 24 h, the 24 h culture was diluted 10-fold into SM sporulation medium (SM broth, Wilson et al., 1982) and anaerobically incubated at 37°C for 48 h. On the day of infection (day 1, 48 h post-inoculation of SM broth), the OD of the SM broth culture was adjusted to an absorbance (OD600nm) of 1 (∼1.0E+09 CFU/mL) in pre-reduced SM broth, and hamsters were orally infected with 0.5 mL of the ∼1.0E+09 CFU/mL (∼5.0E+08 CFU), and the OD adjusted suspension was 10-fold serially diluted in pre-reduced TGY and spot plate onto TSA + SB (5%) to confirm the input CFU per animal. The OD-adjusted suspension (∼1.0E + 09 CFU/mL) was ethanol shocked by diluting 0.2 mL of the suspension into 0.2 mL of ethanol and the mixture was incubated at room temperature for 60 min. The mixture was cold-centrifuged (4°C) for 5 min at 10,000 × *g*. The supernatant was decanted and the cellular/spore pellet was suspend in 0.4 mL of sterile 1X PBS and centrifuged as previously described. The supernatant was decanted and the cellular/spore pellet was suspended in 0.2 mL of sterile 1X PBS. The ethanol shocked mixture was 10-fold serially diluted in pre-reduced TGY and spot plated onto TSA+SB (5%) to determine percentage of spores relative to vegetative cells.

TABLE 4 | cHCDAD study design.

Group	Test article(s)	Regimen[a]	Route	Dose (mg/Kg)	*n*
1	OG157 (Phe17Leu)	TID × 5 days	Ileal canula	20	6
2	OG253 (Phe1Ile)	TID × 5 days		20	6
3	OG168 (Tyr20Phe)	TID × 5 days		20	6
4	OG179 (Phe1Leu)	TID × 5 days		20	6
5	OG161 (Asn18Ala)	TID × 5 days		20	6
6	OG191 (Arg13Asn)	TID × 5 days		20	6
7	Vancomycin	QD × 5 days		20	6
8	Infection (Vehicle) Control	TID × 5 days		NA	6

[a]*Animals were infected with spores on day 1, treated with clindamycin on day 2 (at 24 h after infection), and formulations were administered starting on day 3 (18 h after clindamycin injection) for 5 consecutive days (days 3 through 7). Animals were observed until the end of the relapse period, through day 21. See Section "Materials and Methods" for additional details.*

Clindamycin and Treatment

On day 2, at 24 h after infection, all animals received a single subcutaneous injection of clindamycin (10 mg/Kg). Variant formulations were administered at 20 mg/Kg, three times a day (TID), starting on day 3 (18 h after clindamycin injection) for 5 consecutive days (days 3 through 7) through the surgically implanted ileal canula. Vancomycin (positive control) was prepared in water for injection (WFI) at 50 mg/mL and stored at 4°C over the course of the study. Variant formulations and vancomycin were diluted fresh each day to the appropriate concentration and administered at 20 mg/Kg on days 3 through 7 via the canula and the infection control group was dosed with Test Article vehicle in the same manner. All Test Articles was dosed with ≤0.2 mL volume followed by a 0.1 mL WFI rinse.

Study Design

The design of this study is detailed in **Table 4**. All Test Articles and Vehicle Control were dosed with 0.19 mL volume followed by a 0.1 mL WFI rinse. The identity and concentration of test articles were kept blinded during the study, and 5 individual aliquots of 4.1 mL/tube were provided frozen at ≤−70°C. Each tube, containing sufficient volume for one full day of dosing 6 animals TID with ∼20% excess, was thawed at the morning dose and kept refrigerated (5°C) between doses. Following the last dose of the day, the remaining solution was returned to ≤−80°C storage. Following completion of the study, all tubes containing test articles were returned to Oragenics for testing, to confirm stability, purity and activity (data not shown).

Monitoring

Animals were observed a minimum of three times a day for the duration of the experiment, and more often as appropriate based on clinical condition. General observations included signs for mortality and morbidity, for the presence of diarrhea ("wet tail"), overall appearance (activity, general response to handling, touch, ruffled fur) and were recorded. Body weights were measured and recorded every other day. At the same time, temperatures were measured using a hand-held infrared thermometer for each animal and also recorded. Animals judged to be in a moribund state were euthanized by CO_2 inhalation. Criteria used to assign a moribund state were extended periods (5 days) of weight loss, or progression to an emaciated state, or prolonged lethargy (more than 3 days), or signs of paralysis, or skin erosions or trauma, or hunched posture and a distended abdomen. Observations were continued, with any deaths or euthanasia recorded, for a period up to 21 days post-infection in order to identify animals suffering a relapse of *C. difficile* infection.

Endpoint and Collected Samples

The cecal contents from all hamsters that died during the study, or from those hamsters euthanized by CO_2 inhalation at the end

of the observation period (day 21), were collected and aliquoted into two samples. One sample was used for *C. difficile* TOX AB test (tgcBIOMICS GmbH, Bingen, Germany) and the other stored frozen at −80°C for use in total (spore and CFU) viable counts.

Samples were collected by isolating the cecum through an incision made in the right peritoneal (abdominal) region. The ileocecal junction was carefully exposed by removing the surrounding mesentery and adipose tissue. The ileocecal section of each animal was observed, recorded and photographed. Using minimally serrated forceps, the cecum was picked up so that the end was positioned above the animal. The cecal wall was carefully punctured immediately placed into an open 14-mL Falcon tube. After the contents drained into the tube, the cecal contents were diluted with an equal volume of sterile 1xPBS. The ileum and cecum were removed and stored and tested for CFU/spores as presented above.

Data Analysis

Data was analyzed using GraphPad Prism 6.0d. Survival was compared using Logrank test and Gehan-Breslow-Wilcoxon test. Statistical significance was set at $p < 0.05$.

RESULTS

Compound Selection Strategy

Thirty-two compounds were selected from a saturation mutagenesis library of 418 single amino acid variants of MU1140 (Kers et al., unpublished). An average of 19.5 mg (0.67 mg – 47.1 mg) of each compound was isolated and purified, to >90% purity by flash chromatography (Kers et al., unpublished). Each compound was subjected to a battery of tests designed to identify a reasonable number of lead compounds for animal testing. The overall triage strategy is illustrated in **Figure 2** which included (a) *in vitro* susceptibility assay, (b) *in vitro* toxicity assessment, (c) characterization of chemical stability, and (d) characterization of metabolic stability. The top 6 overall performers from the *in vitro* assays were ultimately tested to evaluate their *in vivo* efficacy, in order to ultimately select a lead compound.

Characterization

The identity of purified compounds was characterized by LC/MS. The dominant peak of 31 compounds confirmed the expected mass (**Supplementary Table S1**). However, Leu6His was characterized by a 23 Da lower mass than expected (calculated mass: 2288.62 Da; observed mass: 2264.8 Da). The cause of the apparent 23 Da mass difference from the expected value is not known. Minor product peaks that appeared common to several variants were identified as the deletion of Phe1. Ten out of 32 variants had a Phe1 deletion. Minor products were also observed with several variants, such as oxidation (+16 Da) and dehydration (−18 Da). It was speculated that the sulfur atom in Ser5Met was oxidized (+16 Da) during manufacturing. The loss of 18 Da would support the concept that the Thr from Leu6Thr and Arg13Thr underwent dehydration (−18 Da) during post-translational modification to give Leu6Dhb and Arg13Dhb,

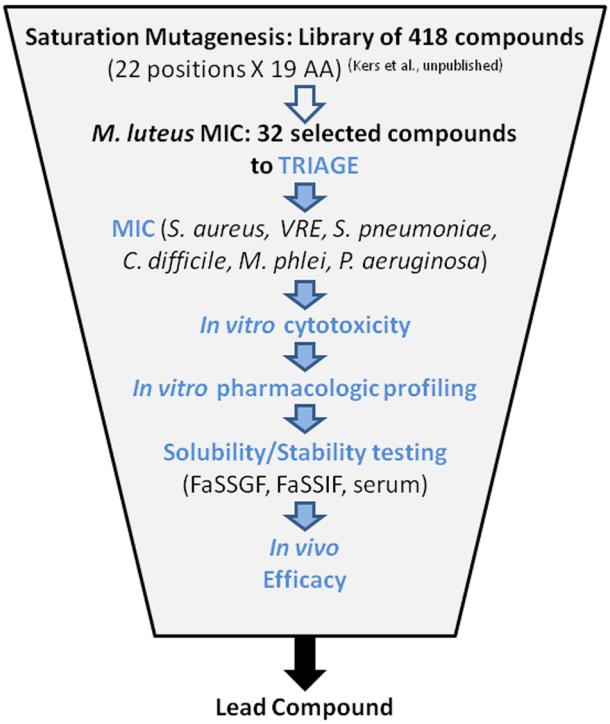

FIGURE 2 | Triage strategy.

respectively (Kers et al., unpublished). The lead compound (OG253, aka MU1140-Phe-1Ile) was further characterized to confirm its primary sequence by extended NMR determination (Smith et al., 2000).

In Vitro Susceptibility Profile

To assess the *in vitro* susceptibility profile and characterize the spectrum of activity of selected variants of MU1140, the MIC was tested against a small panel of representative Gram positive bacterial pathogens. The species selected included *C. difficile* ($n = 3$), VRE ($n = 3$), *Staphylococcus aureus* ($n = 5$), *Streptococcus pneumonia* ($n = 1$) and *Mycobacterium phlei* (as s surrogate for *M. tuberculosis*, $n = 1$). *Pseudomonas aeruginosa* ($n = 1$) was also selected as an example for Gram negative organisms. *M. luteus* was used as a control to monitor consistency with previous reports. The complete MIC dataset is presented in **Supplementary Table S1**. Because of the small sample size used with all other bacterial species, only the MIC data on *S. aureus* allowed for meaningful mode and range calculation. Some degree of variability between strains was observed. In general though there was good consistency in the detected activity of a compound across the panel of Gram-positive organisms tested; a compound that was relatively potent with *M. luteus* was also relatively potent with other Gram positive species tested, with a few exceptions. In contrast, all compounds were equally ineffective against *P. aeruginosa*. Several compounds with adequate MIC against one or several bacterial species were identified and prioritized in subsequent

testing. In particular, at least seventeen (17) compounds were identified with equal or superior *in vitro* susceptibility profiles against *C. difficile*, compared to vancomycin (range 0.5 – 4): Phe1Ile, Phe1Leu, Phe1Tyr, Lys2Ile, Lys2Met, Ser5Met, Leu6His, Leu6Val, Arg13Ala, Arg13Leu, Arg13Asn, Arg13Thr, Gly15Ser, Phe17Leu, Phe17Tyr, Asn18Ala, and Tyr20Phe. One compound (Lys2Met) was identified with a superior i*n vitro* susceptibility profile against VRE, compared to Linezoid, but the significance of this data point was questioned considering the relatively low amounts and mass accuracy of material available for testing (0.67 mg). A few compounds were isolated with similar range as vancomycin against *S. aureus* (0.125 – 2), including Phe1Ala, Leu6His, Phe17Tyr, and Tyr20Phe. Not a single compound was identified with equal or superior MIC to *S. pneumoniae* as compared to ampicillin (MIC = 1.0). The *in vitro* susceptibility profile of *M. phlei* ranged 2 – 16 overall and was inferior to vancomycin (MIC = 0.25). Many compounds that were relatively potent with *M. luteus* demonstrated a lower relative *in vitro* susceptibility profile with other species. While this may have served as a rationale basis for deprioritization, their further characterization was still deemed useful and pursued to investigate whether their MIC could be correlated with other characteristics.

In Vitro Cytotoxicity

A monolayer of a human immortalized liver cell line (HepG2) was used as a first-pass, high-throughput, and inexpensive system to assess potential overt signs of cellular toxicity. Cytotoxicity was assessed using a neutral red uptake bioassay (MB Research Laboratories). The mean percentage viability was determined for each compound tested and compared to vehicle-treated control. A positive result was determined by a greater than 25% decrease in percentage of vehicle-treated cells. When possible, the concentration at which cell viability was reduced to 50% (EC_{50}) was calculated. The complete dataset is presented in **Supplementary Table S1**. The vehicle-alone formulation did not negatively impact the viability of HepG2 cells (data not shown). Interestingly, cytotoxicity was undetectable (maximum concentration tested = 1,000 μg/mL) for approximately half of the compound tested (14 of 32 compounds, or 44%). The remainder had variable levels of toxicity reflected by their EC_{50} between 171 μg/mL and 805 μg/mL (μM range). The overall range of cytotoxicity remains approximately 3 orders of magnitude higher than the expected therapeutic range (low μM to high nM).

In Vitro Pharmacological Profiling

The complete *in vitro* pharmacology profiling dataset as investigated with the Safety Screen 44^{TM} is presented in **Supplementary Table S1**. Altogether, the results showed no consistent target hits in this library of compounds. The profiles were more indicative of low level of random, non-specific inhibition, based on unique structural attributes of individual lantibiotic variants. The data indicate that this class of molecules has limited off-target interactions based on their pharmacological profiles. The data also suggests that no single amino acid variants of MU1140 presents with a drastically different pharmacologic profile relative to MU1140. Mutacin 1140 was positive for only 3 of the 44 assays, each falling within a different functional family. The positive hits included a G protein-coupled receptor (GPCR, delta2 DOP), an ion channel receptor (NMDA) and a kinase (CTK). The amplitude of the signal obtained 42, 25, and 47% respectively, indicative of weak to moderate effects for all targets. Those targets were also found positive with several other variants besides MU1140 (see **Supplementary Table S1** for details). A total of 15 of the 44 targets returned a hit for all MU1140 variants tested (all levels of signal considered). Promiscuity analysis enabled benchmarking of the overall pharmacology profile of MU1140 variants against a library of known compounds (Bowes et al., 2012). Defining the target hit rate as the percentage of targets with >50% inhibition (**Supplementary Table S1**) demonstrate that all MU1140 variants showed a low level of promiscuity (between 0 and 5% of targets are considered selective). It is noteworthy to mention that all high impact targets tested demonstrated a low hit frequency. For example, Ser5Ala was the only variant with a positive hit for hERG (23%, insignificant); the muscarinic acetylcholine receptor M1 had no positive hits; the cyclooxygenase Cox-1 and Cox-2 only had 9 and 1 positive hits, respectively, but they were all considered insignificant (<25% inhibition). The potential correlation between cytotoxicity on HepG2 cells and the *in vitro* pharmacology profiling was investigated, but failed to reveal a positive or negative correlation (data not shown).

Metabolic Stability

Therapeutic peptides are inherently unstable and prone to both chemical and metabolic degradation. The physicochemical environment that a therapeutic peptide encounters depends on the route of administration. The inherent chemical/metabolic stability was characterized for a subset of compounds: (1) by degradation analysis, (2) in two biologically relevant models that simulate the gastrointestinal (GI) environment, and (3) in serum. The contribution of proteolytic degradation was also tested in the biologically relevant simulated fluids by addition of a cocktail of enzymes found in the GI tract. The subset of compounds selected represented compounds with similar *in vitro* susceptibility profiles, cytotoxicity and pharmacological profiles, but anticipated to likely show different stability profiles to chemical and/or proteolytic degradation based on key structural differences. **Table 3** summarizes the stability data that was obtained from these studies. The stability in fasted state simulated gastric fluid (FaSSGF) was consistently high for all compounds tested. In contrast, the stability in fasted state simulated intestinal fluid (FaSSIF) was highly variable amongst the compounds tested, reaching low levels with certain compounds (e.g., Phe17Leu was almost entirely degraded at the completion of this assay). Interestingly, several compounds were identified that presented a better FaSSIF profile than MU1140, suggesting that their resistance to proteolytic degradation could be improved in the GI tract by a single amino acid substitution (e.g., Phe1Ile, Arg13Asn and Tyr20Phe). The stability profile in serum paralleled the profile in FaSSIF.

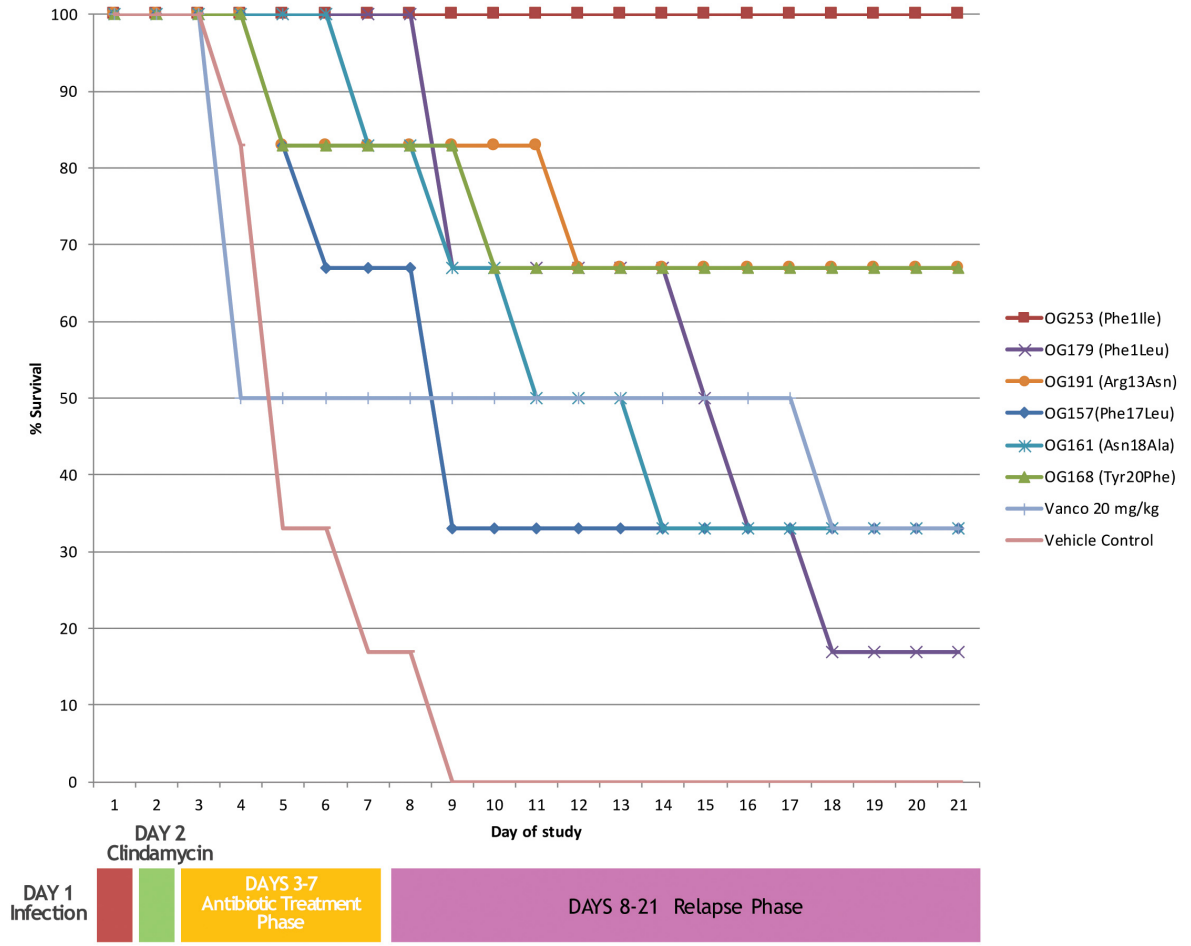

FIGURE 3 | *In vivo* efficacy of top performers. Animals were infected on day 1 followed by clindamycin treatment on day 2. Antibiotics treatment was from days 3–7 and relapse was monitored from until day 21.

Efficacy Assessment *in Vivo*

The top 6 selected compounds were tested in the cannulated Syrian hamster efficacy/relapse model of *C. difficile* infection (cHCDAD). Efficacy was tested in a cannulated model to minimize the potential premature proteolytic or acid hydrolysis-based degradation in the upper GI tract and the results are presented in **Figure 3**. The vehicle controls demonstrated 100% mortality by day 9, while vancomycin-treated animals showed 33% survival at the end of the study period on day 21. In contrast, the lead compound (MU1140-Phe1Ile, aka OG253) showed 100% survival (p = 0.0005 versus vehicle control), following 5 days TID dosing. The other selected compounds displayed varying levels of efficacy ranging from 17% – 67% survival. Tyr20Phe and Arg13Asn exhibited comparable efficacy with 67% survival each by the end of the study on day 21. The remaining test articles, Phe17Leu, Phe1Leu and Asn18Ala, were slightly less effective with 33 – 67% survival on day 9 and 17 – 33% survival for all 3 test articles by day 21. Statistical analysis [Logrank (Mantel-Cox) and Gehan-Breslow-Wilcoxon] of the Kaplan–Meier plots for survival of test article versus vehicle controls groups indicated statistical significance ($p < 0.05$) for all MU1140 variants, except for the Phe17Leu variant (Logrank p = 0.0523, Wilcoxon p = 0.0553). The small number of animal used to support efficacy does not allow for a statistically siginficant effect of relapse to be substantiated when compounds were compared to untreated (n = 1 at day 8) or vancomycin-treated animals (n = 3 at day 8), where only one of the animal relapsed after the treatment phase (after day 8) for each of the control groups. It is noteworthy to mention that relapse in OG253-treated animals was not observed and that a statistically significant effect on relapse was found for OG253-treated animals (n = 6 at day 8) when compared to OG161-treated animals (n = 5 at day 8, Logrank p = 0.0315, Wilcoxon p = 0.0339) or compared to OG179-treated animals (n = 6 at day 8, Logrank p = 0.0041, Wilcoxon p = 0.0061). Post-study analysis of the cecum contents of OG253-treated animals found no detectable *C. difficile* spores (≤1.62Log CFU/g) or Toxin A or B (≤0.27 ng/g) compared to appreciably higher levels detected in vehicle controls (4.09 log CFU, 1061 ng/g Toxin A and 848 ng/g B) and in morbid hamsters (see **Supplementary Figures S1**, **S2**).

DISCUSSION

The compounds selected in the present study were previously engineered via saturation mutagenesis (Kers et al., unpublished). From the initial library of 418 compounds generated, 32 presented with MICs superior to or in the same range as MU1140 against the sensitive indicator strain *Micrococcus luteus*. The current report further characterizes a subset of those 32 top performing compounds, with the ultimate goal of identifying lead compounds for subsequent clinical testing. The triage strategy was designed to be inclusive of desirable attributes of an antimicrobial such as a high *in vitro* susceptibility (low MIC), a low toxicity profile and adequate physicochemical properties (stability and solubility). There are arguably several approaches that can be utilized to fulfill this objective, but the approach favored in this report took advantage of high-throughput screening that could be performed at a relatively low cost with a large amount of pertinent information useful for identifying a manageable number of leads for animal studies.

Considering the intrinsic MIC profile of MU1140 (Ghobrial et al., 2009), finding a variant with similar MIC is desirable, but not necessarily critical to assure an appropriate therapeutic profile, provided other important pharmacological criteria are met. The panel of human bacterial pathogens selected was by no means exhaustive, but selected based on the high incidence, prevalence, morbidity and/or mortality of the associated diseases, as well as previously reported spectrum of activity and *in vitro* susceptibility profile of MU1140 (Ghobrial et al., 2009). The initial selection of *M. luteus* proved adequate for initial screening of MU1140 compounds as it was confirmed that most compounds that were relatively potent against *M. luteus* were also relatively potent against other Gram-positive species tested. In general, the MICs were consistent with previous reports on MU1140 (Ghobrial et al., 2009) and relatively similar within Gram-positive organisms, except that the MIC of several lantibiotic compounds against *C. difficile* appeared superior compared to other bacterial species, based on the limited number of strains tested. The MIC data on *P. aeruginosa* was consistent with previous reports on MU1140 (Ghobrial et al., 2009) and continue to support the spectrum of activity of MU1140 and its variant compounds against Gram positive organisms. A few compounds were identified with superior *in vitro* susceptibility profiles against VREs and/or *S.* aureus, compared to MU1140, linezolid and vancomycin. In contrast, no compounds were identified with equal or superior MIC as compared to MU1140, ampicillin or vancomycin against *S. pneumoniae* or *M. phlei*. Of particular interest was the identification of at least 17 compounds with equal or superior *in vitro* susceptibility profiles as compared to MU1140 and vancomycin against *C. difficile*. While the large number of potential lead candidates against *C. difficile* may have served as a rationale basis for prioritization of this subset of compounds against *C. difficile* associated diseases (CDAD), additional characterization of all 32 compounds was pursued in order to investigate whether their MIC profile could be correlated with other pharmacological or physicochemical properties.

Hepatotoxicity is the most common reason cited for withdrawal of an approved drug (Xu et al., 2004). Unfortunately, there is no simple solution that exists to adequately predict such adverse events *in vitro* (Xu et al., 2004). The limitations of HepG2 cells were particularly well illustrated in a retrospective study of marketed pharmaceuticals (Xu et al., 2004). The data collected to assess cytotoxicity on an immortalized hepatic cell line suggest that MU1140-related compounds are not significantly cytotoxic, or that this assay is not sufficiently sensitive to detect hepatotoxicity. The low levels of toxicity of MU1140 have previously been observed on different cell lines (Oragenics, unpublished) so the former conclusion appears most logical. It is noteworthy to mention that the low levels of cytotoxicity that were observed at high concentrations with a few compounds which remains approximately 3 orders of magnitude higher than the expected therapeutic concentration.

Recognizing the limitations of *in vitro* cytotoxicity assays in general, the Safety Screen 44^{TM} was used to gather insight on potential off-target pharmacological toxicity of selected compounds. This panel allows the gathering as much *in vitro* pharmacologic toxicity information as possible, in a cost-effective panel that brings together both robustness (each assay is HTS-compatible) and the strategic choice of information-rich targets. This system uses a collection of classical competition binding assays and enzymatic inhibition assays with human recombinant proteins for off-target profiling, aiming at identifying undesirable off-target activities that could potentially hinder or halt the clinical development of a compound. The targets chosen in the Safety Screen 44^{TM} fall into 5 functional classes: G protein-coupled receptors (GPCRs), ion channels, enzymes, transporters and nuclear receptors. This profiling panel has been successfully used by leading pharmaceutical companies and provides early identification of significant off-target interactions for the optimization of safety margins (Bowes et al., 2012). While it is evident that the Safety Screen 44^{TM} is a rational early step in the drug discovery process, it was surprising to note that the current report is the first one, to our knowledge, to take advantage of this tool to characterize lanthipeptides in order to assess their "drugability." Promiscuity analysis of the 44 targets tested in the Safety Screen 44^{TM} failed to identify consistently strong, important or obvious patterns, based on variations of MU1140 structure. A direct correlation between promiscuity scores and propensity for toxicity is well documented (Bowes et al., 2012) and also applies to high impact target tested such as hERG, the muscarinic acetylcholine receptor M1 and the cyclooxygenases Cox-1 and Cox-2. That a consistent effect of several MU1140 variants on a receptor system was not found provides no immediate concerns for the class that would provide directional information to collect toxicity data in a specific body system. The data does, however, provide some degree of confidence to assess pharmacotoxicity *in vivo* without pre-identified interactions of concern. Interestingly, a weak to moderate effects were observed with MU1140 and a few variants, including a GPCR, delta2 DOP, an ion channel receptor (NMDA) and a kinase (CTK). The potential pharmacological consequence of the interaction of MU1140 with these receptors is unclear, but would only be expected to have a potential toxicological effect in instances where MU1140 came in direct contact with tissues of the nervous system or cells of the immune system, and at the anticipated

therapeutic dose. Although of low risk, and largely depending on the intended indication and route of administration, further investigation of those specific off-target interaction may be granted in animals and during first-in-human clinical studies. Altogether, the cytotoxicity and pharmacological screens support the concept that MU1140-derived compounds have low toxicity and limited (if any) off-target interactions when administered orally. The data also suggests that no single amino-acid variants of MU1140 presents with an overall different pharmacologic profile, as compared to MU1140.

As a new chemical entity progresses toward the clinic, its stability characteristics increase in importance. On one hand, an antibiotic is expected to meet strict chemical stability criteria during manufacturing and storage, throughout clinical testing and commercialization. On the other hand, the metabolic stability of a compound can directly impact its PK/PD and overall efficacy. Because peptides are generally recognized as being relatively less stable compared to small molecules, we included this aspect in our testing matrix in an effort to identify variants of MU1140 that would present a desirable stability profile without negatively impacting the MIC of the compound. Peptides are generally sensitive to oxygen, moisture, and pH extremes. They tend to form side products by oxidation, deamidaton, dehydration, hydrolysis and β-elimination (Vlasak and Ionescu, 2011). Based on our LC/MS data, low levels of oxidation were observed with Ser5Met. The acid-stability profile of MU1140 and its variants was consistent with previous reports and anticipated (Ghobrial et al., 2009). In contrast, the stability in simulated intestinal fluids (FaSSIF) was highly variable amongst the compounds tested, reaching high levels of degradation for several compounds. Nevertheless, several compounds were also identified that presented with a better FaSSIF profile than MU1140, suggesting that the stability profile could be improved with single amino acid substitutions (e.g., Phe1Ile, Arg13Asn and Tyr20Phe). Others have previously identified Arg13 as being prone to proteolytic degradation and have proposed that Arg13Asp may be a substitution capable of inhibiting proteolytic degradation, while conferring a better MIC (Chen et al., 2013). Unexpectedly, the stability profile in serum paralleled, for the most part, the profile in FaSSIF. From this data, it is evident that MU1140 would be expected to remain very stable under the acidic conditions of the stomach, as evidenced by the unmeasurably high levels of stability in FaSSGF. This data also suggests that none of the amino acid substitutions tested affected this inherent property of MU1140. In contrast, the stability in FaSSIF was highly variable amongst compounds, but could clearly be improved with single amino acid substitutions to the point that the resulting compound was unmeasurably stable in this assay. It was concluded from the stability data that the compounds could be ranked according to their half-life: (a) in FaSSIF: Arg13Asn > Tyr20Phe > Phe1Ile > Phe1Leu > MU1140 > Asn18 Ala > Phe17Leu; (b) or in serum: Phe1Ile > Tyr20Phe > Arg13Asn > MU1140 > Phe1Leu > Asn18Ala > Phe17Leu.

Because of the difficulty in identifying *in vitro* assays that may be predictive of *in vivo* efficacy, the top 6 performers in the *in vitro* assays presented above were further tested in animals. We initially focused on *C. difficile* in CDAD as a model of infection because of the relatively better *in vitro* susceptibility profile of the MU1140-variant compounds to this organism. Cannulation was deemed necessary to optimize the time of contact of the compounds with *C. difficile* infected tissues, and to rule out potential complications related to dissolution, transit time, non-specific adsorption, etc. Further, it allowed the side-by-side testing of compounds that were known to be particularly sensitive to trypsin and chemotrypsin degradation (Arg13 of MU1140), which was present in 5 of the 6 compounds tested. This Golden Syrian Hmster model has previously been used to assess the efficacy of *C. difficile* CDAD (Weiss et al., 2014) and is the current standard *in vivo* model used to assess the potential efficacies of agents, including antibiotics, toxin antibodies, and vaccines (Douce and Goulding, 2010; Weiss et al., 2014). In this model, the vehicle control animals demonstrated 100% mortality by day 9, while vancomycin-treated animals are treated with sub-optimal dose targeting 30-60% mortality in the same time frame to ensure the sensitivity of the test system. From this *in vivo* testing, it can be concluded that all six compounds tested showed some signal of efficacy. Nevertheless, a lead compound emerged based on %-survival at day 21: Phe1Ile (100%) > Arg13Asn and Tyr20Phe (66%) > Asn18Ala and Phe17Leu (33%) > Phe1Leu 17%. It is interesting to note that the spore counts and toxin levels (measured post-mortem) paralleled the clinical outcomes. Specifically, OG253-treated animals had no detectable *C. difficile* spores (≤2 Log CFU/g) or Toxin A or B (≤0.27 ng/g) compared to appreciably higher levels observed in vehicle controls (4.09 log CFU, 1061 ng/g Toxin A and 848 ng/g B) and in morbid hamsters. Taken together, this finding may have significant implications on the problem of recurrence with *C. difficile* CDAD.

Clostridium difficile and other Gram positive organisms have naturally developed tolerance mechanisms against lantibiotics and other cationic antimicrobial peptides (CAMPs) as an evolutionary response to their ecological niche. These mechanisms include increasing the net positive charge of the cell wall or cell membrane, proteolytic degradation, sequestration, export through efflux pumps, the development of biofilms, immune mimicry, etc. (reviewed in Draper et al., 2015). While tolerance mechanisms have been described for lantibiotic producing strains, only low levels of bonafide resistance have been reported for lantibiotics compared with therapeutic antibiotics; resistance phenotypes have been mostly obtained in the laboratory in order to investigate this phenomenon (Draper et al., 2015). For example, the induction of the *cpr*ACB operon *in vitro* decreases the susceptibility levels of *C. difficile* against several CAMPs (McBride and Sonenshein, 2011; Suárez et al., 2013). However, there is no data available supporting that these genes are actually induced *in vivo* or even relevant to the development of lantibiotic-resistance in humans at levels that would cause these molecules to become ineffective therapeutics. For example, a modest 2–4 fold MIC increase of *C. difficile* to gallidermin to ∼1 μg/ml levels (McBride and Sonenshein, 2011) does not imply that a strain harboring this resistance phenotype would be refractory to gallidermin therapy. While mutants of increased MIC may spontaneously arise at the *cpr* locus (McBride and Sonenshein, 2011), there is no data available on the frequency of such mutation under biologically relevant conditions, nor any

data to support that these type of mutations would be selected for in the GI tract of mammalians to a comparable extend as vancomycin-resistant strains of enterococci that are emerging in the clinic (Ahmed and Baptiste, 2017). It is noteworthy to reiterate that nisin resistance was never found to be related to modifications of Lipid II after over 50 years of use as a food preservative.

CONCLUSION

This study is the first of its kind to compare a relatively large collection of lantibiotic variants of MU1140 engineered with single amino acid substitutions. These variants were tested for several key properties, in order to define the most "drugable" of those compounds. This triage strategy supported the selection of the top six performers from a library of 418 variants, based on *in vitro* assays, then to a single lead compound (OG253, MU1140-Phe1Ile) based on animal efficacy studies. Of particular clinical relevance, CDI relapse was not observed in hamsters treated with OG253 at the conclusion of the study. While the animal model used is designed to primarily assess efficacy, the data obtained offer some insight on the potential of OG253 to also affect recurrence in patients, based on the absence of relapse that was observed in OG253-treated animals. It was interesting to note that the one *in vitro* assay that paralleled the *in vivo* efficacy data with the greatest accuracy was the stability in serum. It is unclear whether this is an observation with general applicability. The clinical development of OG253 as a *C. difficile* antibiotic will ultimately impinge on the availability of an appropriate formulation that can assure delivery to the distal portion of the ileum and/or colon. Recent advances in enterically coated capsules, micro-encapsulation and other technologies lead us to believe that this is an achievable goal (reviewed in Felton and Porter, 2013; Luo et al., 2016).

AUTHOR CONTRIBUTIONS

JK, RS, AD, JX, MP, and WW designed and executed the experiments, and reviewed the manuscript. JP analyzed the data and drafted the manuscript. MH conceptualized, designed the experiments, supervised the study, analyzed the data, and prepared the manuscript.

FUNDING

This study was supported by Oragenics, Inc. and Intrexon Corp. performed the molecular biology and strain engineering, data collection and the initial data analysis.

ACKNOWLEDGMENTS

We are grateful to Seventh Wave Laboratories and Almac for performing some of the solubility and stability testing. JP is grateful to the NMR facility at the University of Florida (Dr. Ion Ghiviriga) and Peptide International (Dr. Patricia Y. Coxon) for extended NMR study and Elemental Analysis, respectively. Former employees of Oragenics are acknowledged for their contribution to the work described in this report, including Albert G. Fosmoe, II, Dr. Bruce Tilley, Dr. Gabriela Philips, Dr. Mathoor (Siv) Sivaram, Joel Ngoje, Fernando Anasco, Zaxton Lamon, Emily Richeson, and Vicky DaSilva, who collectively contributed to the success of this program. We are grateful to Dr. Alan Joslyn for critical review of the manuscript, and wish to thank Vince Salvo (ProBiora Health) for assistance with graphic design.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00415/full#supplementary-material

FIGURE S1 | Bacterial titers in intestinal tissues. **(A)** Ileum, **(B)** Caecum, and **(C)** Colon.

FIGURE S2 | Toxin A and B titers at the outcome of the relapse phase or at euthanasia. Blue bar is Toxin A and Red bar is Toxin B. Limit of Detection (LOD) is 1.6 mg/mL.

TABLE S1 | Complete dataset for triage testing.

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Conflict of Interest Statement: JX, MP, and WW had a financial interest in this study as Oragenics sponsored work performed in their respective laboratories. JK and RS, were Intrexon employees during the data collection and initial data analysis. AD was an Oragenics stock-holder and employee during data collection and initial data analysis. JP and MH had a financial interest in Oragenics as stock-holders and employees during the data collection, analysis, and writing of the manuscript.

Part of the data contained in this report was presented at the American Society for Microbiology (ASM) Conference on Antibacterial Development held in Washington, DC, United States, on December 11–14, 2016, and is included in pending US applications 62/362,788, 62/362,809, 62/420,328, and PCT/US 17/42206.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 September 2018
doi: 10.3389/fmicb.2018.02125

Antimicrobial Effect of Asiatic Acid Against *Clostridium difficile* Is Associated With Disruption of Membrane Permeability

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Antibiotic resistance is a major concern in *Clostridium difficile*, the causative agent of antibiotic-associated diarrhea. Reduced susceptibility to first- and second-line agents is widespread, therefore various attempts have been made to seek alternative preventive and therapeutic strategies against this pathogen. In this work, the antimicrobial properties of asiatic acid were evaluated against *C. difficile*. Asiatic acid displayed substantial inhibitory effects on 19 *C. difficile* isolates collected from different sources with minimal inhibitory concentrations ranging from 10 to 20 μg/ml. Time kill analysis and minimal bactericidal concentration revealed potential bactericidal activity of this compound. Asiatic acid induced membrane damages and alterations in morphological ultrastructure in *C. difficile*, thereby causing the leakage of intracellular substances. Moreover, asiatic acid also displayed an inhibitory effect on cell motility, but did not interfere with biofilm formation and spore germination. Analysis of drug combination showed no synergistic effect between asiatic acid and vancomycin/metronidazole. Altogether, asiatic acid exhibited strong antimicrobial activity against vegetative cells and could serve as an alternative resource for tackling *C. difficile*.

Keywords: ***Clostridium difficile*, asiatic acid, antimicrobials, drug resistance, herb**

OPEN ACCESS

Edited by:
Henrietta Venter,
University of South Australia, Australia

Reviewed by:
Nagendran Tharmalingam,
Alpert Medical School, United States
Permal Deo,
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *01 March 2018*
Accepted: *20 August 2018*
Published: *07 September 2018*

Citation:
Harnvoravongchai P, Chankhamhaengdecha S, Ounjai P, Singhakaew S, Boonthaworn K and Janvilisri T (2018) Antimicrobial Effect of Asiatic Acid Against Clostridium difficile Is Associated With Disruption of Membrane Permeability. Front. Microbiol. 9:2125. doi: 10.3389/fmicb.2018.02125

INTRODUCTION

Clostridium difficile infection (CDI) is considered as a major leading cause of infectious diarrhea among hospitalized patients. Over the past decades, there has been a progressive increase in the prevalence and mortality of CDI cases worldwide (Martin et al., 2016). Although patients with CDI may develop the disease from hospitals, potential sources of CDI in humans may include domestic and farm animals since an overlap between isolates from humans and animals has been demonstrated (Janvilisri et al., 2009). As a consequence of long-term antibiotic use, normal gastrointestinal biota is disrupted, allowing scarce population of *C. difficile* to overgrow and colonize in the gastrointestinal tract. The pathogenicity of *C. difficile* depends mostly on the toxins A and B. Both toxins induce the disruption of tight junctions of colonic epithelial cells, causing various symptoms ranging from mild diarrhea to severe pseudomembranous colitis (Voth and Ballard, 2005; Kuehne et al., 2010). Vancomycin and metronidazole are normally prescribed for the patients with CDI according to the clinical guideline, however, 25% of the cases continue to suffer from recurrence. In recent years, significant reduction in the susceptibility of *C. difficile* against

vancomycin and metronidazole has been demonstrated in clinical isolates, causing treatment failure for CDI (Peláez et al., 2002, 2008; Freeman et al., 2010; Tickler et al., 2014). This could potentially be due to the fact that the *C. difficile* develops defensive mechanisms against these prescribed drugs (Harnvoravongchai et al., 2017; Ngernsombat et al., 2017). Although alternative approaches such as fecal transplantation and phage therapy have been introduced for treatment of CDI, however, limitations due to the immunological concern has been addressed (Bakken et al., 2011; Brandt et al., 2012; Sangster et al., 2014). Hence, novel therapeutic options are still in a critical demand. At present, herbs and natural products have regained tremendous attention to be used as promising alternatives for the treatment of bacterial infection. In fact, various active natural extracts against *C. difficile* have been reported, however, the identification of bioactive lead compounds and characterization of their mode of action are still lacking (Aljarallah, 2016; Finegold et al., 2018). Asiatic acid (AA) is a pentacyclic triterpenoid derived from a tropical plant *Centella asiatica*, which has been widely used in traditional remedy in Asia. AA has been shown to exhibit the beneficial effects not only for anti-cancer and neuroprotective activities (Krishnamurthy et al., 2009; Wu et al., 2017), but also displays antimicrobial activity against certain Gram-positive and Gram-negative pathogenic bacteria (Djoukeng et al., 2005; Wong et al., 2011; Liu et al., 2015). However, there is still no report regarding the effect of AA against *C. difficile*. This study therefore aims to investigate the potential inhibitory effect and the mode of action of AA against *C. difficile*.

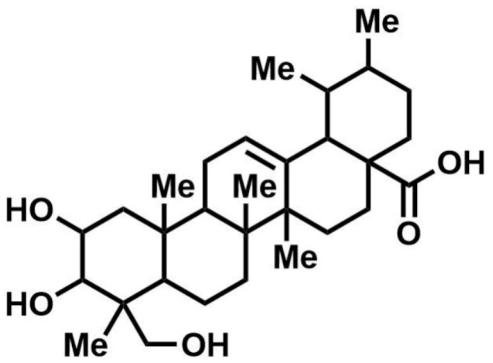

FIGURE 1 | Chemical structure of asiatic acid. Figure was created using ChemDraw version 17.0.0.206 (121). Active code: 683F-0185-F588-C2F2.

MATERIALS AND METHODS

Bacterial Strains, Growth Condition and Chemicals

Clostridium difficile strains 630 and R20291 were kindly provided as a gift from Prof. Nigel Minton, University of Nottingham. Human isolates (RA) of *C. difficile* were previously isolated from diarrheal patients admitted to Ramathibodi Hospital, Thailand during 2010–2011 (Chankhamhaengdecha et al., 2013). The *C. difficile* NIH isolates were obtained from the National Institute of Health (NIH), Thailand. Food and animal *C. difficile* isolates were previously obtained (Ojha et al., 2016). All isolates were cultivated in brain heart infusion (BHI) medium at 37°C under anaerobic condition, unless otherwise mentioned. Asiatic acid with the purity of 97% was purchased from Sigma-Aldrich (St. Louis, MO, United States). The structure of AA is shown in **Figure 1**. The stock AA solution was prepared at 10 mg/ml in dimethyl sulfoxide (DMSO).

Determination of Minimal Inhibitory Concentrations and Minimal Bactericidal Concentrations

Minimal inhibitory concentrations (MICs) were determined by broth dilution method. One percent of overnight bacterial culture was transferred into pre-reduced BHI broth, followed by incubation at 37°C for 8–10 h to avoid the cells entering spore stage. Cell density was diluted to the final inoculum of approximately 3×10^5 CFU/ml in 96 well-plate containing antimicrobial agents. Following 48 h of incubation, optical density at 600 nm was measured using a microplate reader (Tecan). To determine the minimal bactericidal concentrations (MBCs), cell suspensions in 96 well-plates used for MIC test were replicated onto BHI agar. Following the incubation at 37°C under anaerobic condition for 48 h, further bacterial growth on the agar was visually observed.

Time-Kill Analysis

The starter was prepared by growing *C. difficile* strains 630 and R20291 culture overnight. The inoculum was done by transferring one percent of starter culture into BHI broth and incubated for 8-10 h to avoid cells entering spore stage. Time kill assays were carried out with the initial inoculum at approximately 10^8 CFU/ml. Bacterial growth kinetics was followed against different concentrations of AA. The ratio of the absorbance at 600 nm measured at the time *t* and time zero was plotted against time.

Fluorescent Microscopic Analysis

Clostridium difficile strain R20291 suspension was prepared as described above. Cell suspension was treated with 20 μg/ml AA for 0, 2, 4 and 6 h. The samples were then washed, mounted on glass slides, and fixed with methanol. To distinguish live and dead bacterial cells, fixed samples were stained with 500 mM propidium iodide (PI), washed and subsequently counterstained with 1 μg/ml Hoechst 33342. Each sample was visualized and analyzed under FV3000 confocal laser scanning microscope (Olympus). To investigate the cell permeability, *C. difficile* cells were treated with 20 μg/ml AA for 0, 30, 60, and 120 min. The samples then were processed as mentioned previously. After the cells were fixed, each sample was stained with 4′,6-diamidino-2-phenylindole (DAPI), washed and subsequently stained with membrane dye, FM 4-64 (Life technologies). Images were acquired in confocal laser scanning microscope LSM800 with airyscans (Zeiss).

Determination of Protein and Nucleic Acid Leakage

Five ml of *C. difficile* strain R20291 cells at an optical density of 1.0 were harvested and resuspended in pre-reduced phosphate buffer saline (PBS, pH 7.4). AA was added to a final concentration of 40 μg/ml, the solution was then incubated at 37°C for 6 h under anaerobic condition. Cell debris and pellet were removed by centrifugation, and soluble fraction was retrieved for protein determination using Bradford assay and visualized on SDS-PAGE. For the measurement of nucleic acid leakage, total nucleic acids were purified from the soluble fraction by phenol-chloroform extraction, followed by ethanol precipitation. Quantification of the amount of nucleic acid was monitored by spectrophotometer and further verified by agarose gel electrophoresis.

Scanning Electron Microscopy

The effect of AA on cell morphology and ultrastructure of *C. difficile* strains 630 and R20291 was observed by scanning electron microscopy (SEM). *C. difficile* suspension was incubated with AA at 37°C for 6 h, and the suspension was collected and resuspended in PBS pH 7.4. The bacterial cells were fixed with the buffer containing 2.5% glutaraldehyde and 4.0% paraformaldehyde for 4 h, followed by post fixation with a cross-linking reagent, 1.0% osmium tetraoxide, for 1 h. The samples were then dehydrated with a series of ethanol, critical point dried through carbon dioxide, followed by sputter coating with platinum-palladium. The specimens were visualized under Hitachi 2500 scanning electron microscope.

Biofilm Formation and Degradation

One percent of *C. difficile* strain R20291 suspension (Abs_{600} of 0.5) was inoculated into 16 × 150 mm screw capped glass culture tube, containing 5 ml BHI supplemented with 0.1 M glucose. The culture was grown for 5 days to allow biofilm development. Different concentrations of AA were added into the bacterial biofilm, followed by further incubation for 24 h. Remained biofilm was measured with crystal violet staining as described previously with slight modifications (Dawson et al., 2012). The samples were washed twice with PBS to remove unbound cells, subsequently stained with 0.2% crystal violet for 30 min, and washed twice with PBS. Absolute methanol was used to extract crystal violet from the biofilm and the solution was monitored at the absorbance of 570 nm.

Evaluation of Bacterial Motility

The effect of AA on swimming motility of *C. difficile* strain 630 was determined on soft agar. Two microliter of *C. difficile* culture was stabbed into pre-reduced 0.4% agar BHI medium with different concentrations of AA, followed by the incubation at 37°C for 48 h under anaerobic condition. Swimming motility was quantified by measuring diameter of the spot on soft agar.

Spore Preparation and Germination Kinetics

Overnight culture of *C. difficile* strains 630 and R20291 was plated onto 70:30 sporulation medium (Fimlaid et al., 2013), followed by the incubation for 3–4 days. Spores were then harvested as previously described (Ojha et al., 2016). Briefly,

TABLE 1 | Antimicrobial activity of asiatic acid, vancomycin, and metronidazole against *C. difficile* isolated from different sources.

Strains	MIC (μg/ml)			MBC (μg/ml)	Sources
	Asiatic acid	Vancomycin	Metronidazole	Asiatic acid	
R20291	10.0	1.0	0.5	10.0–20.0	Human
630	10.0–20.0	2.0	0.5	10.0–20.0	Human
Fd001	10.0	8.0	16.0	10.0	Food
Fd002	10.0	4.0	8.0	10.0	Food
Fd003	10.0	2.0	8.0	10.0	Food
Bv001	10.0	2.0	8.0	10.0	Animal
Bv002	10.0	2.0	8.0	10.0	Animal
Sw001	10.0	4.0	8.0	10.0	Animal
Ct001	10.0	2.0	8.0	10.0	Animal
RA037	10.0	4.0	2.0	10.0	Human
RA044	10.0	1.0	0.5	10.0	Human
RA156	10.0–20.0	1.0	1.0	10.0–20.0	Human
RA376	10.0	1.0	1.0	10.0	Human
NIH001	10.0	1.0	1.0	10.0	Human
NIH011	10.0	2.0	1.0	10.0	Human
NIH017	10.0	8.0	4.0	10.0	Human
NIH028	10.0	8.0	16.0	10.0	Human
NIH042	10.0	4.0	16.0	10.0	Human
NIH031	10.0	2.0	16.0	10.0	Human

At least 3 biological replicates were performed to ensure the reproducibility.

sporulation induced lawns were collected in distilled water using sterile scarper and washed in distilled water for 5 times. The suspensions were treated with 0.3 mg/ml proteinase K for 1 h, then subjected to heat at 65°C for 1 h to allow efficient inactivation of vegetative cells. Spores were additionally washed with distilled water for 5 times to remove cell debris. Phase contrast microscopy was used to examine the spore purity and spore viability was checked on the agar plate, before being stored at room temperature. To investigate the germination kinetics, germination assay was evaluated as previously described with a few modifications (Bhattacharjee et al., 2015). Spore suspensions were heat-activated at 65°C for 30 min, then subsequently chilled on ice. Activated spores were diluted in BHI supplemented with 0.1% taurocholate. Spore germination was tracked by monitoring the loss of Abs_{600} at 1 min interval for 1 h. The ratio of the Abs_{600} at the time *t* and time zero was plotted against time.

Drug Combination Assay

Bacterial susceptibility to the combination of AA and either vancomycin or metronidazole was tested by checkerboard assay (Magi et al., 2015). Two-fold serial dilutions of AA at the final concentrations ranging from 0.625 to 40 μg/ml were mixed with either vancomycin at a range of 0.25–16 μg/ml or metronidazole at a range of 0.03125–2 μg/ml in 96-well plates. Approximately 3×10^5 CFU/ml of *C. difficile* strains 630 and R20291 were inoculated, followed by the incubation at 37°C for 48 h under anaerobic condition. To determine the drug combination effect, fractional inhibitory concentration (FIC) index was calculated according to the equation: FIC index = $FIC_A + FIC_B$, where $FIC_A = MIC_{A+B}/MIC_A$ and $FIC_B = MIC_{B+A}/MIC_B$. MIC_{A+B} is the MIC of compound A in the combination with compound B and *vice versa* for MIC_{B+A}, whereas MIC_A or MIC_B are the MIC of the compound alone. The combination effect is defined as synergy when FIC $\leq$ 0.5, additive when 0.5 $<$ FIC $\leq$ 1.0, no interaction when 1.0 $<$ FIC $\leq$ 4.0, and antagonism when FIC $>$ 4.0 (Páez et al., 2013).

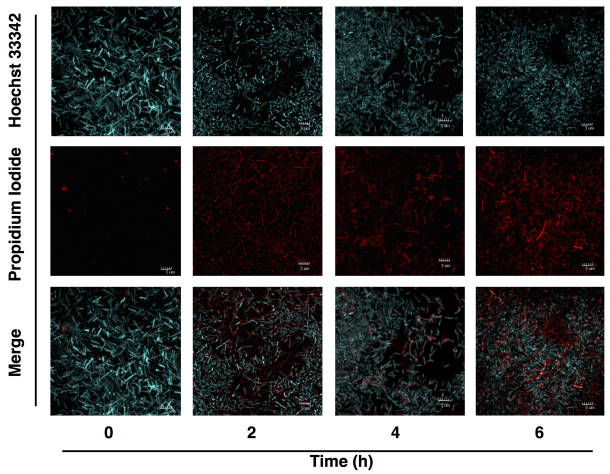

FIGURE 3 | Viability of *C. difficile* following treatment with asiatic acid. Fluorescent images were captured after *C. difficile* strain R20291 was exposed to AA at 0, 2, 4, and 6 h. Blue-stained bacteria (Hoechst 33342) represented live cell, while red-stained bacteria (propidium iodide) represented dead cell.

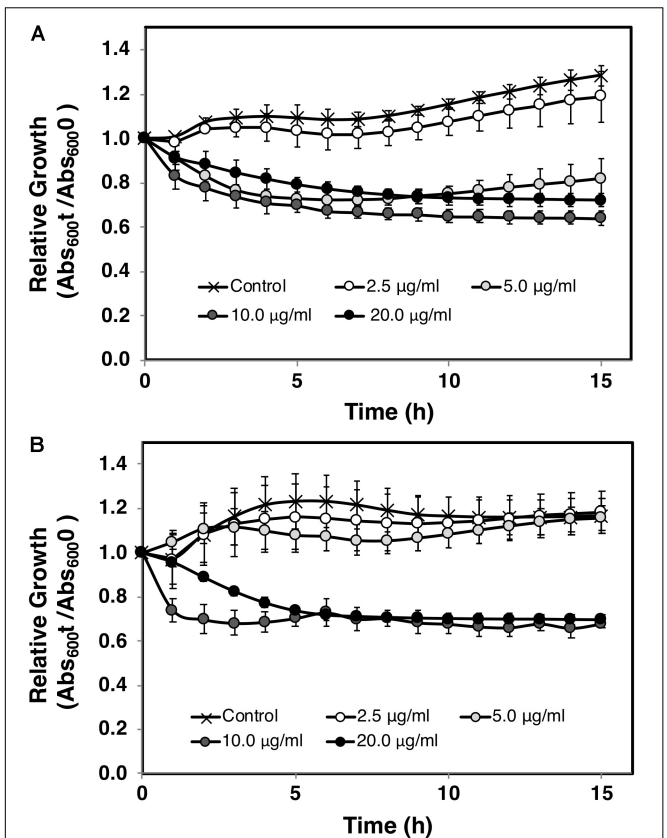

FIGURE 2 | Time kill curve of *C. difficile* treated with asiatic acid. *C. difficile* strains **(A)** R20291 and **(B)** 630 were grown in BHI broth medium with various concentrations of asiatic acid. Growth kinetics was followed for 15 h at 1 h interval. 1% DMSO was added to the control sample. Mean values of at least 3 independent measurements (± standard error) are plotted.

Statistical Analysis

Unless indicated otherwise, all statistical analyses were based on at least 3 independent experiments. The statistical analyses were performed using Student's *t*-test with a 95% confidence interval for the sample mean. The *p*-value less than 0.05 indicates statistically significant difference.

RESULTS

Asiatic Acid Inhibits the Growth of *C. difficile*

An inhibitory effect of AA on 19 *C. difficile* strains isolated from different sources was investigated by determining the MIC values. The result of MICs of AA and recommended antibiotics used for CDI treatment including vancomycin and metronidazole were summarized in **Table 1**. AA exhibited substantial inhibitory effect against *C. difficile* strains with the MIC value of 10.0 μg/ml, excepted for human isolates 630 and RA156, whose MIC

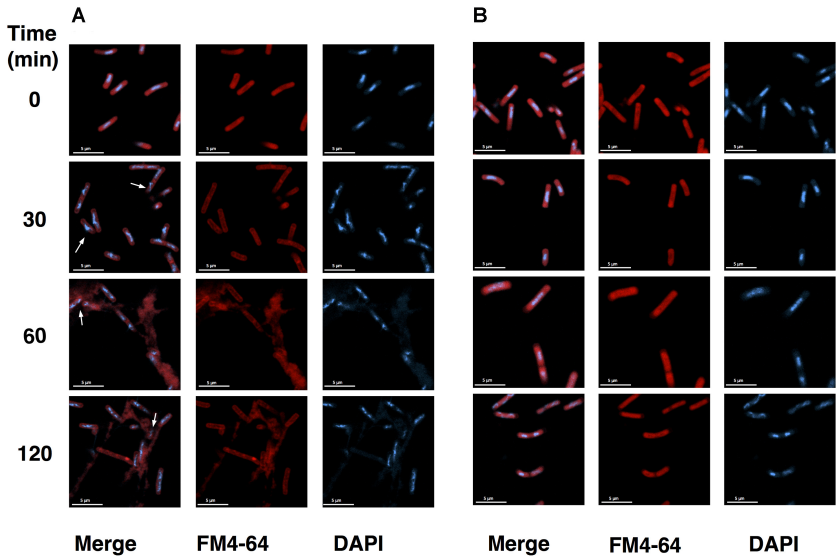

FIGURE 4 | *C. difficile* membrane integrity is compromised in the presence of asiatic acid. Fluorescence microscopic images of **(A)** *C. difficile* strain R20291 treated with 20 μg/ml AA at each time interval and **(B)** untreated samples stained with membrane dye FM4-64 (Red) and DNA dye DAPI (Blue). Red fluorescence indicates membrane disintegration (permeable membrane). Scale bar: 5 μm.

values were 20.0 μg/ml. On the contrary, a broad range of MICs of vancomycin and metronidazole against the tested *C. difficile* strains was evident. The MICs of vancomycin ranged from 1.0–8.0 μg/ml, while the MICs of metronidazole were from 0.5–16.0 μg/ml. MBC assays were concomitantly performed to demonstrate the bactericidal effect of AA on *C. difficile*. According to **Table 1**, MBCs of AA against *C. difficile* was equal to 10.0 μg/ml, except for the strains 630, R20291 and RA156, which MBC values were shifted up to 20.0 μg/ml.

The effect of AA on *C. difficile* growth was assessed using *in vitro* time kill experiments. The relative growth as measured by Abs_{600} at the time t compared to $t = 0$ was plotted against times. An apparent reduction of bacterial viability was seen as indicated by the decline of the slope (**Figure 2**). In agreement with MIC/MBC data, the exposure to AA at the concentrations of 10 and 20 μg/ml totally inhibited the growth of both *C. difficile* strains 630 and R20291. Bactericidal effect of AA was further validated by live and dead cell assays using PI/Hoechst 33342 staining and fluorescent microscopy. PI is a membrane impermeant dye, while Hoechst 33342 exhibits membrane permeability in bacterial cells, therefore only dead cells accumulate and display red intensity. As shown in **Figure 3**, the levels of red intensity increased in a time-dependent manner, implying the higher population of dead cells over the exposure time. These results were strongly consistent with the bactericidal activity indicated in the time kill curve.

Asiatic Acid Disrupts *C. difficile* Membrane Permeability

Changes in bacterial cellular morphology were observed using FM4-64/DAPI staining and fluorescent microscopy. As shown in **Figure 4**, leakage of bacterial DNA was observed in *C. difficile* R20291 following the exposure to AA for 30 min (**Figure 4A**), and the damages to plasma membranes were observed at the exposure time of 60 and 120 min. In contrast, DNA was tightly packed in the intact cells for untreated control condition (**Figure 4B**). The leakage of proteins and nucleic acids in the suspension was also evaluated. Up to 2.5-fold of protein leakage was observed in the cell suspension of *C. difficile* following the AA treatment as shown in **Figures 5A,B**, and the increase was calculated to be statically significant ($p < 0.05$). Similarly, the amount of released nucleic acids in the suspension was significantly elevated to ∼3-fold compared to the untreated controls (**Figures 5C,D**). Alterations in the ultrastructure of *C. difficile* cells following the exposure to AA were observed using SEM analysis (**Figure 6**). While the control cells retained their normal rod-shaped structure with smooth and intact surfaces, the exposure to AA caused damages on the bacterial plasma membrane as indicated by the number of craters on the surface and rupture of cells. The intense of damages was correlated well with the concentration of AA.

Asiatic Acid Inhibits Cell Motility, but Does Not Interfere Biofilm and Spore Germination

The effects of AA on other virulence factors including biofilm, motility and spore germination were investigated. Ability of *C. difficile* to adhere and form biofilm causes an increase in bacterial virulence (Dapa and Unnikrishnan, 2013). Biofilm of *C. difficile* R20291 was grown and subjected to AA. The results revealed that the amount of biofilm was not affected even upon the exposure of high concentration of AA (80 μg/ml) (**Figure 7A**). For cell motility, the significant reduction in colony diameters of *C. difficile* 630 was observed when the concentration of AA reached 10 μg/ml ($p < 0.05$) (**Figures 7B,C**).

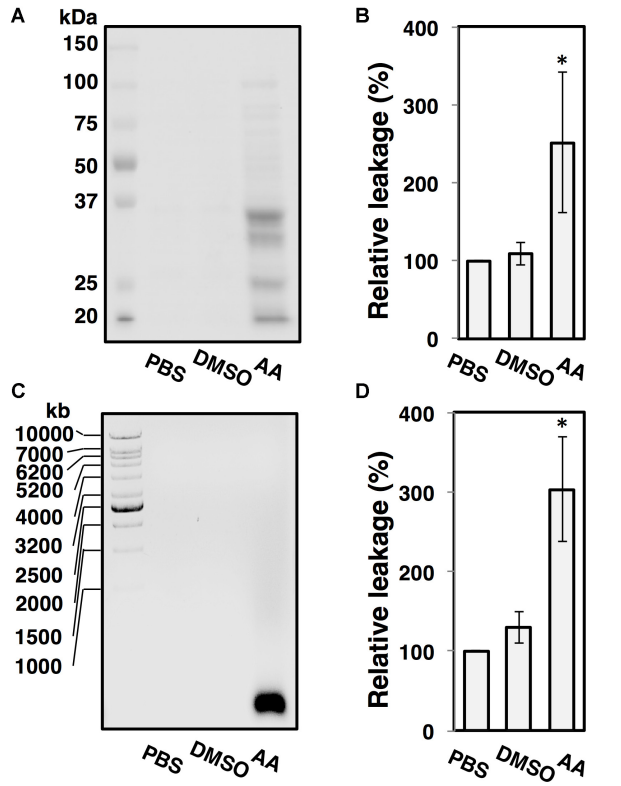

FIGURE 5 | Asiatic acid causes bacterial intracellular component leakage. *C. difficile* strain R20291 grown in BHI medium was anaerobically incubated in PBS buffer, PBS with 1% DMSO and PBS with 40 μg/ml AA. **(A)** Released proteins from the supernatant fractions were analyzed by SDS-PAGE, **(B)** Bar graphs represent the relative protein leakage as determined by Bradford assay, **(C)** Released nucleic acids from the supernatant fractions were analyzed by agarose gel electrophoresis, **(D)** Bar graphs represent the relative nucleic acid leakage as quantified by UV spectrophotometry. At least 3 independent tests were performed to ensure the reproducibility. The error bars and the asterisks represent the standard errors and the statistical significance with the *p*-values < 0.05, respectively.

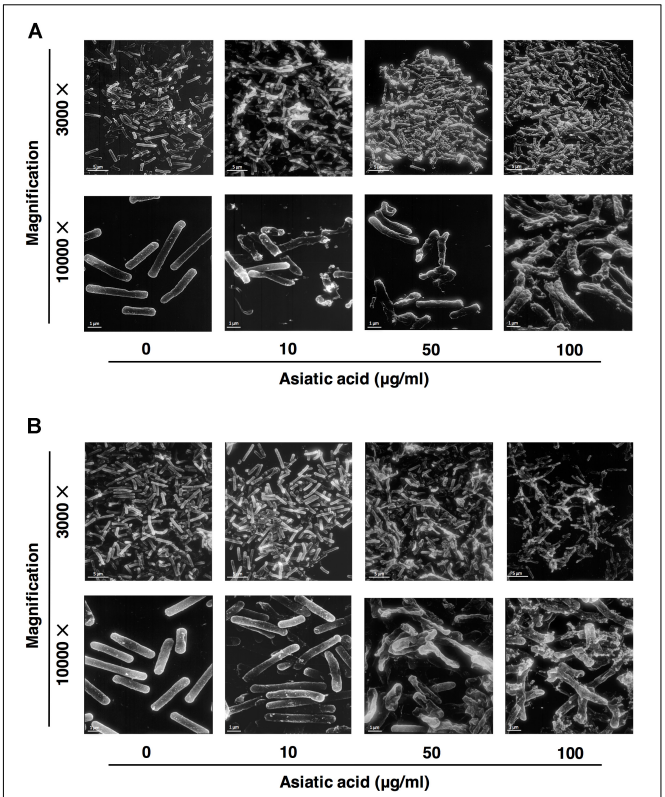

FIGURE 6 | Scanning electron microscopic analysis of asiatic acid-treated *C. difficile* strains **(A)** R20291 and **(B)** 630. Bacterial cells were treated with 10, 50, and 100 μg/ml AA, and were then subjected to SEM. Control (untreated) cells displayed intact smooth surfaces as shown in the left panel. Scale bar: 1, 5 μm.

It should be noted that no colony formation was seen when the concentration of AA exceeded 10 μg/ml. To determine whether AA could inactivate *C. difficile* spore, purified spores of *C. difficile* strains R20291 and 630 were subjected to AA, and germination kinetics was followed by monitoring the changes in Abs_{600} over a 1 h time period. The relative germination rate was calculated along with the increasing AA concentrations. The results revealed no significant changes in spore germination of both strains at any tested concentrations of AA (**Figures 8A–D**). Despite no effect of AA on spore germination, ultrastructure of C. *difficile* R20291 exhibited a few visible damages on the spore surface following the exposure to 10 and 100 μg/ml AA (**Figure 8E**).

Asiatic Acid Exhibits No Synergism With Vancomycin and Metronidazole

The synergistic effect of AA and vancomycin/metronidazole was explored using checkerboard method and the isobolograms were plotted (**Supplementary Materials**). Based on our results, AA exhibited no synergistic effect with both vancomycin and metronidazole as FIC indices were calculated to fall in a range of 1.0 to 1.5, suggesting that AA acted individually with vancomycin or metronidazole.

DISCUSSION

Prior to the era of antibiotic discovery, folk remedies were widely practiced through the combination of traditional knowledge and implementation of herb medication, health prevention and promotion. Despite the fact that current medication is based on the use of antibiotics, a considerable part of the world population still depends on alternative treatment. In many countries, more than 50% of medical prescriptions rely on traditional medicine, meanwhile 38% of adult patients in the United States preferred herbal medicines as an alternative treatment (Benzie and Wachtel-Galor, 2011). AA, an active constituent mostly present ∼0.5% of dry weight in the leave of *Centella asiatica* (Rafamantanana et al., 2009), has been proved to possess several pharmacological effects including anti-inflammatory, anti-aging and anti-cancer properties (Huang et al., 2011; Tsao and Yin, 2015; Ren et al., 2016). AA also

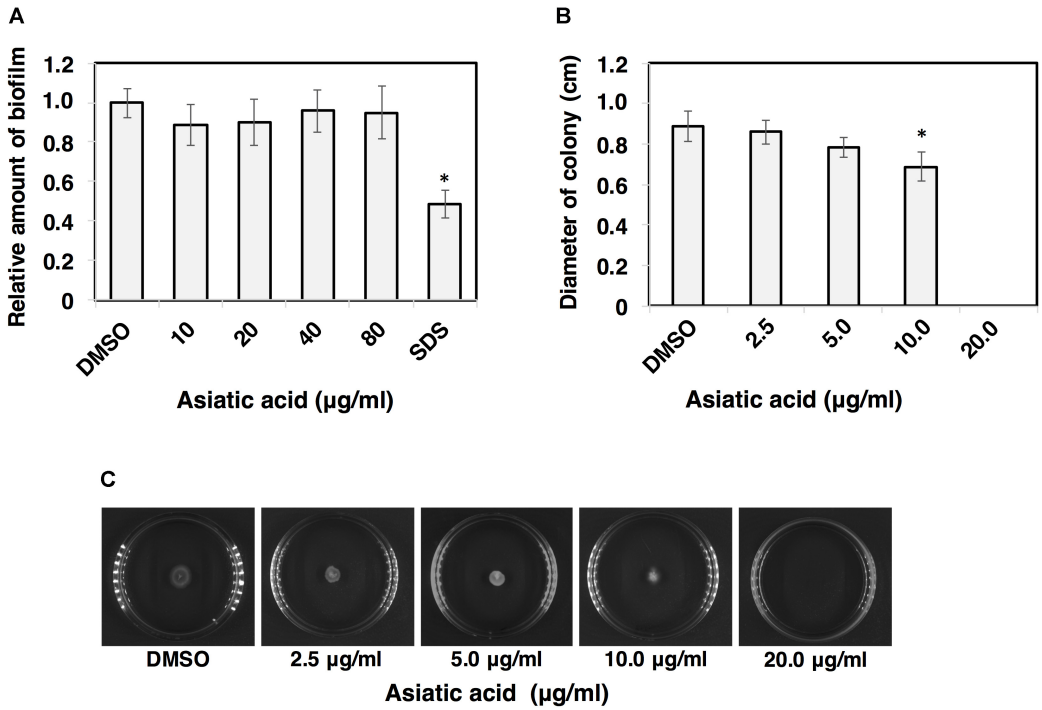

FIGURE 7 | Asiatic acid inhibits *C. difficile* motility, but does not induce biofilm degradation. **(A)** Effect of AA at different concentrations on biofilm degradation in *C. difficile* strain R20291. SDS-treated positive control was included. **(B)** Bar graphs represent migration distance of *C. difficile* strain 630. **(C)** Images represent swimming motility of *C. difficile* strain 630 on BHI soft agar at various concentrations of AA. At least 3 independent experiments were performed. The error bars and the asterisks represent the standard errors and the statistical significance with the p-values < 0.05, respectively.

exhibits an antimicrobial activity against certain pathogenic bacteria (Liu et al., 2015). As the current therapy for *C. difficile* relies on antibiotic treatment including vancomycin and metronidazole, and the resistance to both drugs is becoming more pronounced, hence we attempted to explore the potential use of AA as an alternative therapeutic against *C. difficile*.

Susceptibility tests of *C. difficile* with AA showed consistent MIC value among isolates from different sources (10–20 μg/ml). A lower inhibitory concentration of AA against *C. difficile* was observed compared to the previously reported MICs of 24–40 μg/ml on other pathogens (Liu et al., 2015). Variations could be resulted from differences in tested bacterial species and experimental conditions. In parallel, MIC values of vancomycin and metronidazole, the antibiotics recommended in the practical guideline for treatment of CDI, were also determined. The MICs of these two antibiotics varied among tested strains. The human isolate NIH028 exhibited the highest MIC against vancomycin and metronidazole (8.0 and 16.0 μg/ml, respectively) implying the possibility of the resistance development during the antibiotic treatment. The resistance to vancomycin and metronidazole in *C. difficile* strains isolated from the patients in hospitals has been demonstrated (Peláez et al., 2002, 2008; Baines et al., 2008; Spigaglia et al., 2011; Karlowsky et al., 2012). The food isolate Fd001 also exhibited high resistance to both drugs. Because the transmission of *C. difficile* occurs via fecal-oral route, the emergence of antibiotic resistant isolates from food marks an alarm for critical concerning on the transmission of *C. difficile* in contaminated diet (Lund and Peck, 2015). Although higher concentration of AA was required to inhibit certain *C. difficile* isolates, compared to those in vancomycin and metronidazole, however, constant dosage of the compound used for the inhibition allows the determination of precise dose for alternative treatment. MBCs of AA in all tested isolates ranged between 10.0 and 20.0 μg/ml, which do not exceed 4 times of the MICs, indicating the bactericidal activity of the compound. In accordance with the MBC test, time kill kinetics determined in *C. difficile* strains R20291 and 630 demonstrated a decline in bacterial density as a result of AA exposure. *C. difficile* strain R20291 exhibited higher sensitivity to AA compared to the strain 630, which is in agreement with those observed in MIC test. It should be noted that the kinetic studies were followed for 16 h, while MIC determinations were performed for 48 h, therefore the dissimilarity of the susceptibility profiles could be clearly demonstrated between each time intervals rather than at one end point. An increase in the population of dead cells following the exposure to AA was evidently time-dependent, which further supports the role of AA as a bactericidal agent. Prior to the drug administration, cytotoxicity of the compounds against host cells is needed to be evaluated (Tharmalingam et al., 2018). IC_{50} values for AA were reported to be greater than 100 μM (∼50 μg/ml) on human umbilical vein endothelial cells and kidney epithelial Vero cells (Jirasripongpun et al., 2012; Huang et al., 2016),

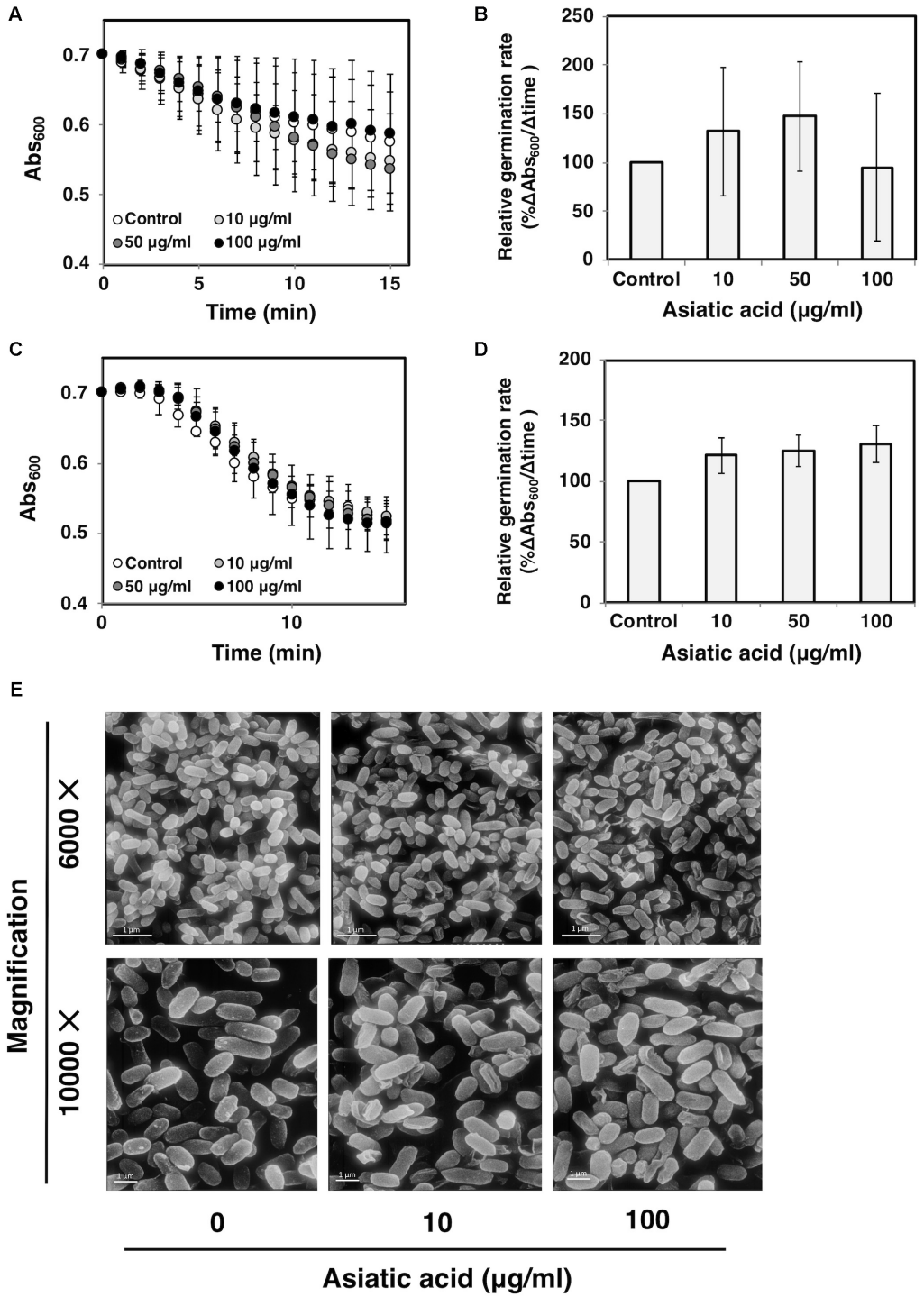

FIGURE 8 | Asiatic acid does not inactivate *C. difficile* spore germination. *C. difficile* spores from strains **(A,B)** R20291 and **(C,D)** 630 were exposed to various concentrations of AA. The germination kinetics was tracked for 15 min at 1 min interval. Bar graphs represent relative germination rate. At least 3 independent tests were performed to ensure the reproducibility. Mean values of at least 3 independent experiments (± standard error) are plotted. **(E)** Scanning electron micrographs of *C. difficile* spores from strain R20291 exposed to 10 and 100 µg/ml AA.

as well as no significant effect of the compound at the same concentration was observed on viability of human gastric mucosa epithelial cells (Jing et al., 2016). Additionally, up to 165 mg/kg of AA was administered to the mice without any signal of side effects (Krishnamurthy et al., 2009). Hence, AA could be a decent therapeutic agent for CDI due to the substantially

low MIC against *C. difficile* compared to the cytotoxicity level.

AA is a terpenoid, an organic compound made up of isoprene units, which is usually found in plant essential oils. It has been demonstrated that terpenoids can cause large-scale membrane thinning on lipid bilayer and could therefore exert their antimicrobial properties via a membrane disruption mechanism (Khandelia et al., 2010). Correspondingly, several studies on terpenoids extracted from plants proposed the properties of the compounds to act on the bacterial plasma membrane, which eventually lead to cell dead (Jasmine et al., 2011; Sathya Bama et al., 2012). To determine the potential mode of action of AA on cell permeability, leakage of intracellular compounds was evaluated. The fluorescence microscopic analyses revealed the rapid action of AA on *C. difficile*, which caused the leakage of DNA from the bacterial cells within 30 min, while the shape of cell was still retained. Distorted bacterial membrane with no enclosed DNA was later visualized upon the longer incubation period, in accordance with the amount of released proteins and nucleic acids quantified in the supernatant. Additionally, severe damages at the surface including cell rupture and alterations in cellular morphology as demonstrated in SEM micrographs of both 630 and R20291 strains were consistent with the damages previously observed in other bacteria (Hartmann et al., 2010; Goldbeck et al., 2014; Hong et al., 2015). These data suggested that AA potentially acts on cell membrane and causes substantial membrane rupture on *C. difficile*. As the integrity of bacterial plasma membrane is crucial not only for cell protection but also houses enzymes responsible for cellular processes such as energy production. Thus, bacterial membrane disruption could collapse cellular processes, which in turn affects *C. difficile* survival and growth.

Ability to motile and adhere involves in the development of disease of *C. difficile* (Twine et al., 2009; Dingle et al., 2011; Stevenson et al., 2015). Thus, we assessed the inhibitory effect of AA on the motility of *C. difficile*. The results showed that the motility of *C. difficile* was significantly reduced following the exposure to AA. Noteworthy, even with a concentration below the inhibitory dose, AA could potentially reduce the infection by impairing colonization and adhesion of *C. difficile*. Previous reports showed that certain naturally derived compounds including anthocyanins, could inhibit toxin secretion via transcription repression in a gastrointestinal pathogen, *Helicobacter pylori,* at the sub-inhibitory concentrations (Kim et al., 2012). Further investigations should be addressed for toxin reduction in *C. difficile*. Following *C. difficile* colonization, biofilm formation could be initiated. Bacterial biofilm is an aggregated form of bacterial community, in which each individual cell is compacted and connected through the released extracellular polymeric substances. It has been shown that biofilm formation increases the resistance to vancomycin up to 10 folds in *C. difficile* (Dapa and Unnikrishnan, 2013), reflecting the consequent complication for antibiotic treatment. Although AA exhibited the strong inhibitory effect on *C. difficile* via the disruption of membrane permeability, degrading activity of the compound was not observed when tested against biofilm. Extracellular polymeric substances are usually composed of different biomolecules such as polysaccharides, proteins, DNA and lipids, which serve as a rigid architecture for bacteria. Hence, AA that is supposed to act on lipid membrane, might not be able to penetrate through the barrier of the complicated extracellular matrix of the biofilm.

Clostridium difficile spores are highly resistant to harsh conditions including heat and toxic chemicals, and play a critical role in the transmission of the disease. Physical and chemical treatments have been reported to inactivate spores and inhibit their germination (Rutala et al., 1993; Perez et al., 2005; Fawley et al., 2007; Francis et al., 2013; Ojha et al., 2016). Here, we also attempted to examine the effect of AA on spore germination. Although AA exhibited an inhibitory effect against *C. difficile* vegetative cells, however, spore germination did not seem to be significantly influenced by the presence of AA, even at high concentration. On the contrary with the germination data, SEM analysis revealed a detectable spore damage when exposed to 10 μg/ml of AA. It could be explained by the fact that (i) only a few spores were damaged, thus the overall germination rate was not affected, and (ii) spores are encoded by several rigid layers (Paredes-Sabja et al., 2014), therefore the damages at the spore surface may not affect spore germination.

Furthermore, synergism between AA and the first- and second-line agents for CDI including vancomycin and metronidazole was investigated. The FIC values of AA and vancomycin/metronidazole fell into the range of 1.0–1.5, suggesting no synergistic effect between AA and these drugs. This might due to the difference in mode of actions as AA is proposed to disrupt bacterial cell membrane, while vancomycin and metronidazole kill bacteria through the inhibition of cell wall and DNA synthesis, respectively (Huang et al., 2009). Considering no antagonistic effect, CDI patients who have been treated with vancomycin or metronidazole, can be alternatively treated with AA without any contravention.

CONCLUSION

Asiatic acid exhibited remarkable antimicrobial activity against *C. difficile* by disrupting permeability of the cell membrane. Nevertheless, the compound was unable to destroy dormant spores or biofilm. Taken into account of the strong inhibition properties of AA and low cytotoxicity level, the compound could be further developed as an alternative treatment to combat CDI.

AUTHOR CONTRIBUTIONS

PH and TJ conceived and designed the study. PH, SC, and PO performed the experiments. SS and KB helped with the experimental assays. PH and TJ wrote the paper. TJ supervised the project. All authors have read and approved the manuscript.

FUNDING

This research project was supported by a grant from the government budget to Mahidol University (TJ), and an initiative research grant from Mahidol University (PH).

ACKNOWLEDGMENTS

We appreciate Methinee Pipatthana and Anupong Bunyacharoenchit for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02125/full#supplementary-material

FIGURE S1 | No synergistic effect between asiatic acid and vancomycin/metronidazole. **(A)** Checkerboard test of *C. difficile* strains 630 and R20291 on BHI medium supplemented with the combination of AA and either vancomycin or metronidazole. Shading area represents visible growth. **(B)** Isobolograms of AA plus vancomycin or metronidazole. Dotted lines represent the additive effect of the drug combination (FIC = 1). At least 3 independent tests were performed to ensure the reproducibility.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PD and handling Editor declared their shared affiliation.

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ORIGINAL RESEARCH
published: 11 June 2018
doi: 10.3389/fmicb.2018.01256

In Vitro Activity of Tedizolid, Dalbavancin, and Ceftobiprole Against *Clostridium difficile*

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Background: *Clostridium difficile* (*C. difficile*) is a major nosocomial pathogen that colonizes in the human gut. Recently, the U.S. FDA approved three new antimicrobial agents against gram-positive bacteria: Tedizolid, Dalbavancin, and Ceftobiprole. The efficacy of these antibiotics for treatment of *C. difficile* infection has not been thoroughly examined. The current study aimed to examine the *in vitro* activity of these antibiotics against *C. difficile*. In addition, to compare between Dalbavancin and Ceftobiprole to antibiotics from the same class: Vancomycin and Ceftriaxone, respectively.

Methods: Eighty-four *C. difficile* isolates were tested for susceptibility to Tedizolid, Dalbavancin, Ceftobiprole, Vancomycin, and Ceftriaxone by Etest technique in order to determine the minimum inhibitory concentration (MIC).

Results: Upon comparison of the novel antibiotic agents, Dalbavancin demonstrated the lowest MIC values and ceftobiprole the highest at MIC_{50} (0.016, 0.38, and 1.5 μg/mL, for Dalbavancin, Tedizolid, and Ceftobiprole, respectively) and MIC_{90} (0.03, 0.78, and 3.17 μg/mL, respectively). Dalbavancin demonstrated significantly lower MIC_{50} and MIC_{90} values compared to Vancomycin (0.016 vs. 0.38 and 0.03 vs. 3.5, respectively) ($p < 0.001$) and ceftobiprole had significantly lower MIC values compare to ceftriaxone (1.5 vs. 32 and 3.17 vs. 28.8, respectively) ($p < 0.001$).

Conclusion: Dalbavancin and Tedizolid may play a role as potential therapeutic agents for treatment of *C. difficile* infection. Examination of antibiotic effect on the intestinal microbiome and clinical trials are needed for more accurate results.

Keywords: *Clostridium difficile*, vancomycin, tedizolid, dalbavancin, ceftobiprole, ceftriaxone

OPEN ACCESS

Edited by:
Santi M. Mandal, Indian Institute of Technology Kharagpur, India

Reviewed by:
Suman Saha, Priyamvada Birla Aravind Eye Hospital, India
Joy Scaria, South Dakota State University, United States

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *17 October 2017*
Accepted: *24 May 2018*
Published: *11 June 2018*

Citation:
Binyamin D, Nitzan O, Azrad M, Hamo Z, Koren O and Peretz A (2018) In Vitro Activity of Tedizolid, Dalbavancin, and Ceftobiprole Against Clostridium difficile. Front. Microbiol. 9:1256. doi: 10.3389/fmicb.2018.01256

INTRODUCTION

Clostridium difficile is a gram-positive rod, an obligate anaerobe, spore forming, toxin-producing bacterium that colonizes the human gut (Hall and O'Toole, 1935). The incidence of *C. difficile* infection (CDI) has increased markedly worldwide since 2000 (Kelly and LaMont, 2008). In Israel, the incidence of *C. difficile* infection in 2016 was 2761 cases per 100,000 patients (Israeli National Center for Infection Control, unpublished data). Disease symptoms include diarrhea and abdominal pain, fever, and increased levels of blood lymphocytes. In severe cases, pseudomembranous colitis, toxic megacolon, or colonic perforation may occur, with high mortality

rates (Johanesen et al., 2015). The highly resistant spores can survive on surfaces for long periods, and thus be easily transferred from person to person. This occurs mainly in hospitals and long-term care facilities; therefore CDI is a nosocomial infection and has important clinical and financial implications for hospitals (Paredes-Sabja et al., 2014).

The major risk factor for CDI is antibiotic administration, which disrupts the normal intestinal microbiota, leading to spore germination and proliferation of *C. difficile*. Although nearly all antimicrobial classes have been associated with CDI, clindamycin, third-generation cephalosporins, fluoroquinolones, and penicillins are most commonly associated with this disease (Owens et al., 2008).

Several treatment strategies exist; in some cases, cessation of antibiotics that induced CDI is sufficient for a cure. However, the majority of patients are treated with antibiotics such as metronidazole and Vancomycin (Davies et al., 2011). In severe and recurrent cases, fecal microbiota transplantation, a treatment that restores the normal fecal microbiota, is efficacious (Youngster et al., 2014).

Recently, the U.S. FDA approved three new antimicrobial agents against gram-positive bacteria: Tedizolid, Dalbavancin, and Ceftobiprole. Tedizolid is an oxazolidinone derivate with higher potency than Linezolid, requiring a lower dose for effective result (Im et al., 2011; Prokocimer et al., 2013; Sahm et al., 2015). Tedizolid is highly active against gram-positive bacteria, and can be used for the treatment of acute bacterial skin and skin structure infections (Ferrández et al., 2017). This antibiotic binds to the 50S subunit of the bacterial ribosome and inhibits protein synthesis (Shaw et al., 2008).

Dalbavancin is a bactericidal lipoglycopeptide antibiotic that inhibits cell wall synthesis. Similar to Vancomycin, it belongs to the glycopeptide antibiotic group and is an efficient treatment for infections caused by Vancomycin-resistant strains (Tatarkiewicz et al., 2016). *In vitro* Dalbavancin activity against various gram-positive species, including *C. difficile*, was more potent compared to Vancomycin (Goldstein et al., 2003; Huband et al., 2016; Tatarkiewicz et al., 2016).

Ceftobiprole is a new generation of cephalosporin with broad-spectrum activity against gram-positive and gram-negative bacteria (Ednie et al., 2007). Ceftobiprole is a β-lactam antibacterial with bactericidal activity (Murthy and Schmitt-Hoffmann, 2008).

The current study aimed to examine the *in vitro* activity of Tedizolid, Dalbavancin, and Ceftobiprole against *C. difficile* by detecting the minimum inhibitory concentration (MIC). In addition, we also compared Dalbavancin and Ceftobiprole to Vancomycin and Ceftriaxone, respectively, antibiotics from the same class.

MATERIALS AND METHODS

Study Population

Patients diagnosed with *C. difficile* infection at the Baruch Padeh Medical Center, Poriya in northern Israel, were enrolled in the study from January 2015 to May 2017. The identification of CDI was performed by stool examination for toxigenic *C. difficile* at the Clinical Microbiology Laboratory by Xpert® *C. difficile* Assay (Cepheid, Solna, Sweden), performed on Cepheid GeneXpert® Systems. This is a qualitative *in vitro* real-time PCR for the rapid identification of *C. difficile*. The study was approved by the Poriya Baruch Padeh Medical Center Helsinki Committee without the need for patients to sign an informed consent form because the study deals with microbial isolates and the results of the study do not affect the patients.

Bacteria Isolation and Identification

For this purpose, 0.5 mL of liquid feces was suspended in 4.5 mL physiological solution. Fifty μL of the suspension were inoculated on a selective CHRomagar medium; chromID™ *C. difficile* (bioMérieux, France) and then incubated at 37°C in anaerobic conditions (GasPakTM EZ, BD, United States) for 48 h. *C. difficile* colonies appear as asymmetric and black-colored colonies. Final identification was done by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based technology using the Bruker Biotyper system (Bruker, United States).

Antibiotic Susceptibility Tests

Antibiotic susceptibility tests were performed by the Etest technique in order to determine the minimum inhibitory concentration (MIC), which is the lowest antibiotic concentration that inhibits bacterial growth. To this end, bacteria colonies were suspended in saline, generating turbidity of 0.5 McFarland. The suspensions were seeded on Mueller Hinton + 5% Sheep Blood agar plate (HyLaboratories, Rehovot, Israel). Then, a gradient Etest strip (Liofilchem, Italy) for each antibiotic was added to each plate and incubated at 37°C in anaerobic conditions for 48 h. After incubation, the susceptibility breakpoint was determined as the lowest concentration at which no bacterial growth was detected. Additionally, MIC_{90} and MIC_{50} were calculated as the MICs at which 90%/50% of the isolates tested are inhibited. Etest procedures were done for Tedizolid, Dalbavancin, and Ceftobiprole, as well as for Vancomycin and Ceftriaxone for comparison to Dalbavancin and Ceftobiprole, respectively.

Statistical Analysis

Differences between the MIC_{50} or MIC_{90} of Tedizolid, Dalbavancin, and Ceftobiprole were analyzed by one-way analysis of variance (ANOVA), with Benferroni's *post hoc* test.

Differences between the MIC_{50} or MIC_{90} of Ceftobiprole and ceftriaxone as well as the differences between the MIC_{50} or MIC_{90} of Dalbavancin and Vancomycin, were analyzed by Wilcoxon matched-pairs signed rank test.

Statistical significance was defined by $p < 0.05$.

RESULTS

Eighty-four *C. difficile* isolates were tested for susceptibility to Tedizolid, Dalbavancin, and Ceftobiprole. MIC_{50} and MIC_{90}

TABLE 1 | Susceptibility test (MICs) of *C. difficile* isolates.

Antibiotic		Tedizolid	Dalbavancin	Ceftobiprole	*p*-Value
MIC (μg/mL)	**Range**	0.032–32	0.002–0.250	0.016–32	<0.001
	MIC_{50}	0.38	0.016	1.5	
	MIC_{90}	0.78	0.03	3.17	

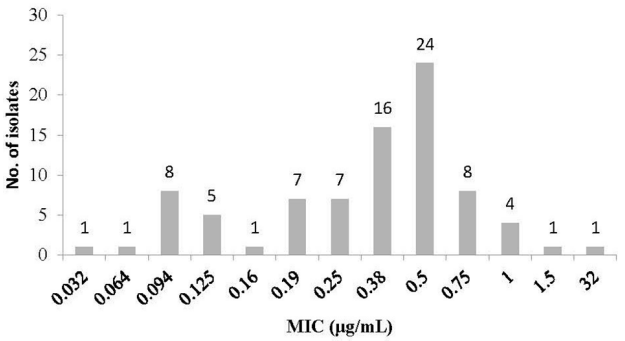

FIGURE 1 | Minimum inhibitory concentration (MIC) distribution of *Clostridium difficile* isolates to Tedizolid.

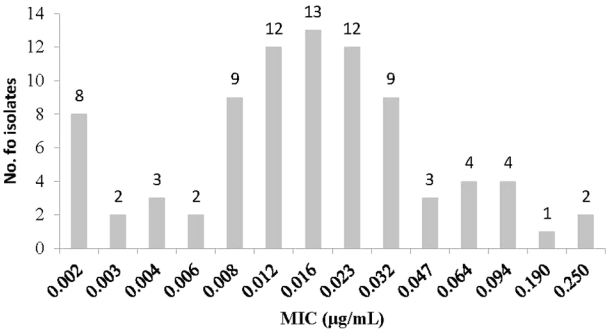

FIGURE 2 | Minimum inhibitory concentration distribution of *C. difficile* isolates to Dalbavancin.

results are presented in **Table 1**. The MIC_{50} and MIC_{90} results of each antibiotic were different from the other antibiotic agents ($p < 0.001$). Dalbavancin demonstrated low MIC results compared to Tedizolid and Ceftobiprole, for MIC_{50} (0.016, 0.38, and 1.5 μg/mL, respectively) and MIC_{90} (0.03, 0.78, and 3.17 μg/mL, respectively). Ceftobiprole was the antibiotic with the highest MIC values compared to the two other antibiotics.

The distribution of MIC for Tedizolid is presented in **Figure 1**. Most isolates had an MIC of 0.5 μg/mL (24 isolates) or 0.38 μg/mL (16 isolates). In addition, some of the isolates had low MIC values (for instance 8 isolates with 0.094 μg/mL and 5 isolates with 0.125 μg/mL). However, other isolates had higher MIC values (for instance 1 isolate with an MIC of 32 μg/mL and 8 isolates with an MIC of 0.75 μg/mL).

Figure 2 presents the MIC distribution for Dalbavancin. Overall, Dalbavancin susceptibility results demonstrated low MIC values. Most isolates had an MIC of 0.016 μg/mL (13 isolates) and 0.012 or 0.023 μg/mL (12 isolates).

Minimum inhibitory concentration distribution for Ceftobiprole is presented in **Figure 3**. Most isolates had an MIC of 1.5 μg/mL (21 isolates) or 2 μg/mL (15 isolates). Many isolates had high MIC values; for instance 8 isolates with MIC of 3 μg/mL, 6 with 4 μg/mL, and 3 with 32 μg/mL.

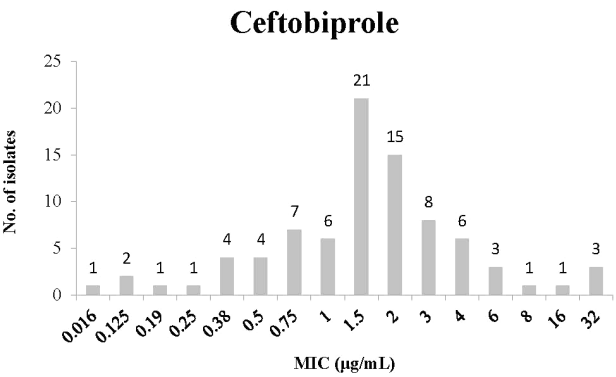

FIGURE 3 | Minimum inhibitory concentration distribution of *C. difficile* isolates to Ceftobiprole.

We compared the susceptibility of antibiotics from the same antibiotic group (**Table 2**). In a comparison of the MIC_{50} and MIC_{90} between Dalbavancin and Vancomycin, Dalbavancin showed significantly lower MIC values (0.016 vs. 0.38 and 0.03 vs. 3.5, respectively, $p < 0.001$).

TABLE 2 | Comparison between susceptibility tests (MICs) of antibiotics in the same antibiotic group.

Antibiotic		Dalbavancin	Vancomycin	*p*-Value	Ceftobiprole	Ceftriaxone	*p*-Value
MIC (μg/mL)	**Range**	0.002–0.250	0.016–256	<0.001	0.016–32	0.38–32	<0.001
	MIC_{50}	0.016	0.38		1.5	32	
	MIC_{90}	0.03	3.5		3.17	28.8	

In comparison of the MIC_{50} and MIC_{90} between Ceftobiprole and Ceftriaxone, Ceftobiprole had significantly lower MIC values (1.5 vs. 32 and 3.17 vs. 28.8, respectively, $p < 0.001$).

DISCUSSION

Antibiotic administration is the main risk factor for CDI, even though treatment of the disease includes antibiotic therapy. Despite the efficacy of the conventional antibiotics for treating the disease, treatment failure, recurrence, and antibiotic resistance are reported (Adler et al., 2015; Freeman et al., 2015). Therefore, it is important to identify new antibiotics that may potentially be used to treat CDI.

The current study examined the MIC of three recently approved antibiotics: Tedizolid, Dalbavancin, and Ceftobiprole. These antibiotics are affective against gram-positive bacteria such as methicillin resistant *Staphylococcus aureus* and Vancomycin resistant *Enterococci* (Ednie et al., 2007; Im et al., 2011; Huband et al., 2016). However, antibiotic efficacy against *C. difficile* has not been thoroughly examined.

Of all three antibiotics, Dalbavancin was found to be the most effective antibiotic with high prevalence of isolates with low MIC values. In comparison to Vancomycin, another lipoglycopeptide antibiotic, Dalbavancin, had significantly lower MIC values for *C. difficile*. These results are different from those found in a previous study, where higher MIC levels for Dalbavancin and lower MIC levels for Vancomycin were demonstrated, compared with our findings, but similar to what we found – MIC values for Dalbavancin were lower than those of Vancomycin (Goldstein et al., 2003). Vancomycin is considered first line treatment of moderate to severe CDI (Davies et al., 2011). Different studies reported resistance rates of 7.4–47% to Vancomycin, treatment failure in 14.2% of cases, and recurrence rate of 24.0% (Adler et al., 2015; Freeman et al., 2015; Tkhawkho et al., 2017). Due to the presence of Vancomycin-resistant strains and recurrence cases, Dalbavancin might be used as treatment for CDI. Further research is required, mainly clinical trials and examination of antibiotic effect on the intestinal microbiome.

Ceftobiprole had the highest MIC values for *C. difficile* compared to the other examined antibiotics, although these MIC values were lower than those found in another study (Ednie et al., 2007). In comparison to another broad spectrum cephalosporin, ceftriaxone, *C. difficile* isolates had significantly lower MIC values. Cephalosporins have poor *in vitro* activity against *C. difficile* (Wilcox et al., 2016). Some cephalosporins even promote *C. difficile* spore germination, proliferation, and toxin production (Wilcox et al., 2016). However, a study in a mouse model that tested Ceftobiprole's effect on *C. difficile* found that Ceftobiprole does not promote the growth of *C. difficile* or toxin production, in contrast to Ceftazidime, Cefotaxime, and Ceftriaxone (Nerandzic and Donskey, 2011). Another study that investigated the effect of Ceftobiprole administration on normal intestinal microbiota found that it had no significant ecological impact on the human intestinal microbiota (Bäckström et al., 2010). Consequently, Ceftobiprole can be used for CDI treatment and when administered for other indications may be associated with a reduced risk for CDI compared with other cephalosporins.

We also found a high prevalence of isolates with low MIC values to Tedizolid. This antibiotic was shown to have high activity against gram-positive bacteria, mainly skin pathogens, with low MIC rates (Bensaci and Sahm, 2017; Ferrández et al., 2017). Ours is the first study that examines the effect of Tedizolid on *C. difficile*. In a clinical trial, fewer patients who had received Tedizolid suffered from gastrointestinal side effects (16%) than those who received Linezolid (23%) (Shorr et al., 2015). Further research is warranted to determine whether Tedizolid is effective against *C. difficile*.

CONCLUSION

The activity of three novel antibiotics – Tedizolid, Dalbavancin, and Ceftobiprole – against *C. difficile* was examined. Dalbavancin and Tedizolid may be potential therapeutic agents for the treatment of CDI. Clinical trials are needed to confirm the laboratory experiments, as well as studies that examine the effect of these antibiotics on the intestinal microbiome to ensure they are not a risk factor for CDI.

AUTHOR CONTRIBUTIONS

DB, ON, OK, and AP designed the study and interpreted the data. AP, MA, and ON drafted the manuscript. DB, ZH, MA, and AP performed laboratory work. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 June 2018
doi: 10.3389/fmicb.2018.01206

A Small Molecule-Screening Pipeline to Evaluate the Therapeutic Potential of 2-Aminoimidazole Molecules Against *Clostridium difficile*

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 01 March 2018
Accepted: 17 May 2018
Published: 06 June 2018

Citation:
Thanissery R, Zeng D, Doyle RG and Theriot CM (2018) A Small Molecule-Screening Pipeline to Evaluate the Therapeutic Potential of 2-Aminoimidazole Molecules Against Clostridium difficile. Front. Microbiol. 9:1206. doi: 10.3389/fmicb.2018.01206

Antibiotics are considered to be the first line of treatment for mild to moderately severe *Clostridium difficile* infection (CDI) in humans. However, antibiotics are also risk factors for CDI as they decrease colonization resistance against *C. difficile* by altering the gut microbiota and metabolome. Finding compounds that selectively inhibit different stages of the *C. difficile* life cycle, while sparing the indigenous gut microbiota is important for the development of alternatives to standard antibiotic treatment. 2-aminoimidazole (2-AI) molecules are known to disrupt bacterial protection mechanisms in antibiotic resistant bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus,* but are yet to be evaluated against *C. difficile*. A comprehensive small molecule-screening pipeline was developed to investigate how novel small molecules affect different stages of the *C. difficile* life cycle (growth, toxin, and sporulation) *in vitro*, and a library of commensal bacteria that are associated with colonization resistance against *C. difficile*. The initial screening tested the efficacy of eleven 2-AI molecules (compound 1 through 11) against *C. difficile* R20291 compared to a vancomycin (2 μg/ml) control. Molecules were selected for their ability to inhibit *C. difficile* growth, toxin activity, and sporulation. Further testing included growth inhibition of other *C. difficile* strains (CD196, M68, CF5, 630, BI9, M120) belonging to distinct PCR ribotypes, and a commensal panel (*Bacteroides fragilis, B. thetaiotaomicron*, *C. scindens, C. hylemonae*, *Lactobacillus acidophilus, L. gasseri, Escherichia coli, B. longum* subsp. *infantis*). Three molecules compound 1 and 2, and 3 were microbicidal, whereas compounds 4, 7, 9, and 11 inhibited toxin activity without affecting the growth of *C. difficile* strains and the commensal microbiota. The antimicrobial and anti-toxin effects of 2-AI molecules need to be further characterized for mode of action and validated in a mouse model of CDI.

Keywords: *C. difficile*, small molecules, 2-aminoimidazole, growth, toxin, sporulation

Abbreviations: 2-AI, 2-aminoimidazole; CDC, The Centers for Disease Control and Prevention; CDI, *C. difficile* infection; CPD, cysteine protease domain; MIC, minimum inhibitory concentration.

INTRODUCTION

Clostridium difficile is the leading cause of nosocomial and antibiotic associated infectious diarrhea worldwide. *C. difficile* causes over 450,000 infections and 29,000 deaths annually in the United States (Lessa et al., 2015; McDonald et al., 2018). The incidence, severity, and recurrence rates have increased markedly with the emergence of epidemic strains, and exposure to classic risk factors such as recent antibiotic use, advanced age, and prior hospitalization (Stabler et al., 2006; Ananthakrishnan, 2011; Loo et al., 2011). In addition, *C. difficile* is now increasingly being linked to community acquired cases of colitis in individuals not exposed to typical risk factors (CDC, 2008; Gupta and Khanna, 2014; Knetsch et al., 2017; McDonald et al., 2018). The changing epidemiology, and the subsequent challenges in the treatment of this infection has prompted the Centers for Disease Control and Prevention (CDC) to classify *C. difficile* as an urgent threat to public health (CDC, 2013).

Clostridium difficile infection (CDI) is initiated by spores that are highly resistant to various physical and chemical stressors, enabling them to persist in the environment, and play a key role in disease transmission (Baines et al., 2009; Loo et al., 2011; Deakin et al., 2012; Paredes-Sabja et al., 2014). In the gut, the presence of calcium, glycine, and primary bile acids such as taurocholate sensed by the germinant receptor CspC enables *C. difficile* spores to germinate into metabolically active vegetative cells (Sorg and Sonenshein, 2008; Francis et al., 2013; Kochan et al., 2017). However, the normal indigenous gut microbiota provides colonization resistance against *C. difficile* (Theriot et al., 2014; Buffie et al., 2015). Antibiotic mediated disruption of the gut microbiota and metabolome leads to a loss of colonization resistance favoring vegetative cell proliferation, and production of toxins that ultimately mediate disease (Antunes et al., 2011; Theriot et al., 2016). During CDI, *C. difficile* initiates the sporulation pathway forming metabolically dormant spores there by completing the life cycle. The signals that trigger the onset of sporulation are not well understood, however, substantial evidence supports the link between nutrient limitation or other stress factors with sporulation and virulence (Paredes-Sabja et al., 2014; Nawrocki et al., 2016). Current line of treatment for patients with CDI includes the antibiotics vancomycin, metronidazole, or fidaxomicin, which in approximately 20–30% of the patients is ineffective resulting in recurrence (Cohen et al., 2010; Lessa et al., 2015). The intrinsic damage caused by the current line of antibiotics on the gut microbiota, and its failure to restore colonization resistance is the major limiting factor in the treatment and management of CDI (DuPont, 2011). There are occasional reports of *C. difficile* having high MIC *in vitro* to the drugs used for its treatment (Baines et al., 2008; Martin et al., 2008; Snydman et al., 2012), however, to date treatment failures have not been linked to antimicrobial resistance. Considering the ease with which *C. difficile* spread globally in a short time span (He et al., 2013), coupled with the fact that antibiotics are risk factors, there is growing consensus for drug targets that selectively inhibit *C. difficile* vegetative cells and or virulence factors, while sparing the indigenous gut microbiota. Compounds that inhibit sporulation would also be beneficial as they would aid in the prevention of transmission and relapse.

Identifying potential drug targets against *C. difficile* is challenging because of the complex etiology, and the impact of risk factors that lead to the disease (Smits et al., 2016). Traditionally, MIC's and kill assays were used in initial drug screening pipelines, which focuses only on the growth stage of the *C. difficile* life cycle. Here we present a comprehensive small molecule pipeline, which evaluates the activity of test compounds on three different stages of the *C. difficile* life cycle (growth kinetics, toxin activity, and sporulation), and how they impact the growth of *C. difficile* strains from distinct PCR ribotypes. Additionally, the pipeline evaluates how these small molecules alter the growth of other gut commensals that are associated with colonization resistance against *C. difficile*. The goal of the *in vitro* screening strategy described here is to screen and select promising compounds that are able to inhibit one or all of the steps in the *C. difficile* life cycle. Future work defining the mechanism of action of each compound and validating them in a mouse model of CDI is down stream of this pipeline.

2-aminoimidazole (2-AI) molecules have a unique mechanism of action by targeting two-component systems (TCSs), which are signaling pathways that allow bacteria to respond to environmental signals (antibiotics or quorum sensing molecules) there by inhibiting virulence responses such as antibiotic resistance, toxin secretion, and biofilm formation (Thompson et al., 2012). These processes are important in pathogenesis and survival of the pathogen within the host (Stock et al., 2000; Stephenson and Hoch, 2002; Beier and Gross, 2006). 2-AI molecules have been successfully used for antibiotic potentiation and anti-virulence activities against other antibiotic resistant bacteria such as *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus aureus*, but are yet to be evaluated against *C. difficile* (Rogers et al., 2010; Brackett et al., 2014; Draughn et al., 2017). *C. difficile* relies on TCS signaling pathways for toxin production that mediate disease, and sporulation which plays a key role in transmission and recurrence (Underwood et al., 2009; Darkoh et al., 2015, 2016). Therefore, we hypothesized that 2-AI molecules would be able to inhibit different stages of *C. difficile* life cycle namely toxin activity and sporulation. In this study, we started with eleven 2-AI molecules (compound 1 through 11) in our comprehensive screening pipeline, and tested their ability to inhibit *C. difficile* growth, toxin activity, and sporulation. Molecules that showed potent activity against *C. difficile* R20291 were further tested against other *C. difficile* strains (CD196, CF5, M68, BI9, 630, and M120) belonging to distinct PCR ribotypes, and an eight-member commensal library of bacteria associated with colonization resistance against *C. difficile*. Compound 1, 2, and 3 were found to inhibit growth kinetics, whereas compounds 4, 7, 9, and 11 inhibited toxin activity without affecting the growth of both *C. difficile* strains and commensals. Next steps include evaluation of each compound for the mechanism of action, and validation in a mouse model of CDI.

TABLE 1 | Commensal microbiota library.

Phyla	Bacteria	Strain*	Description	Nucleotide accession no. (complete genome)/Reference
Bacteroidetes	*Bacteroides fragilis*	NCTC 9343	Type strain, appendix abscess	GenBank, CR626927
Bacteroidetes	*Bacteroides thetaiotaomicron*	VPI-5482	Type strain, human feces	Xu et al., 2003
Firmicutes	*Lactobacillus acidophilus*	ATCC 700396/NCFM	Infant feces	Altermann et al., 2005
Firmicutes	*Lactobacillus gasseri*	ATCC 33323	Type strain	GenBank, CP000413
Firmicutes	*Clostridium scindens*	ATCC 35704	Type strain, human feces	GenBank, ABFY02000000
Firmicutes	*Clostridium hylemonae*	TN-271	Type strain, human feces	GenBank, AB023972**
Proteobacteria	*Escherichia coli*	ATCC BAA 2649	Not type strain	
Actinobacteria	*Bifidobacterium longum* subsp. *infantis*	DSM 20090	Intestine of infants	Mattarelli et al., 2008

**ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; DSM, Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures.*
***16S rRNA, partial sequence.*

TABLE 2 | Minimum inhibitory concentration of 2-aminoimidazole molecules against *C. difficile* strain R20291 compared to vancomycin.

Test compound	MIC (μg/ml)
Vancomycin	0.15–0.31
1	2.5–5
2	5
3	5
4	>10
5	>10
6	>10
7	>10
8	>10
9	>10
10	>10
11	>10

Minimum inhibitory concentration (MIC) was determined by broth microdilution as per modified CLSI guidelines for anaerobes. Data represent mean values from triplicate trials.

MATERIALS AND METHODS

Bacterial Strains

Clostridium difficile Strains and Growth Conditions

Clostridium difficile strains selected from a range of PCR ribotypes, including epidemic (R20291 and M68), non-epidemic (CD196, CF5, and 630), current (R20291, M68, and BI9), and a genetically divergent strain (M120) were used in these studies. R20291, CD196, CF5, M68, 630, BI-9, and M120 belongs to ribotypes 027, 027, 017, 017, 012, 001, and 078, respectively. The origin and reference details of the isolates can be obtained from **Table 2** of our previous publication (Sebaihia et al., 2006; Stabler et al., 2009; He et al., 2010; Thanissery et al., 2017). All assays using *C. difficile* were started from spore stocks. Spores were prepared and tested for purity as described previously (Perez et al., 2011; Thanissery et al., 2017). Briefly, individual *C. difficile* strains were grown anaerobically in 2 ml Columbia broth at 37°C for 12 h and further sub-cultured into 40 ml Clospore media in which it was allowed to sporulate for 5–7 days. Spores were harvested by centrifugation and subjected to 3–5 washes with sterile cold water. Spore stocks were stored at 4°C in sterile water until use. The spores were heat treated (65°C for 20 min) to kill vegetative cells, before enumeration and testing for purity. The viable spores were enumerated on brain heart infusion (BHI, Becton, Dickinson and Company, Sparks, MD, United States) media supplemented with 100 mg/L L-cysteine and 0.1% taurocholate. To ensure purity, spores were plated on BHI media plus 100 mg/L L-cysteine, with and without spore germinant (0.1% taurocholate). The purified spores were further examined under phase contrast microscope in which non-germinated intact spores appeared as phase bright bodies. *C. difficile* cultures for the assays were prepared by inoculating spores on BHI media supplemented with 100 mg/L L-cysteine and 0.1% taurocholate. The plates were incubated anaerobically overnight at 37°C, and isolated colonies from these plates were used to prepare *C. difficile* inoculum in BHI broth with 100 mg/L L-cysteine.

Commensal Library Strains and Growth Conditions

Eight different non-*C. difficile* strains that are members of the healthy human gut microbiota belonging to four dominant bacterial phyla including Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were used to determine MIC's of various 2-AI molecules. Strain details and sources are shown in **Table 1**. *Bacteroides fragilis* NCTC 9343, and *Bacteroides thetaiotaomicron* VPI-5482 were obtained from Eric Martens (University of Michigan, United States). *Clostridium hylemonae* TN-271 was obtained from Joson M. Ridlon (University of Illinois Urbana-Champaign, United States). *Lactobacillus acidophilus* ATCC 700396, *Lactobacillus gasseri* ATCC 33323, and *Bifidobacterium longum* subsp. *infantis* DSM 20090 were obtained from Rodolphe Barrangou (North Carolina State University, United States). *Clostridium scindens* (ATCC 35704, Cat # 35704) and *Escherichia coli* (Cat # BAA 2649) were purchased from American Type Culture Collection. All strains were maintained as 15% glycerol stock in −80°C until use. Working stocks of *Bacteroides species* were prepared in tryptone-yeast extract- glucose (TYG) media (Martens et al., 2008). *C. scindens, C. hylemonae,* and *E. coli* were grown in BHI

plus 100 mg/L L-cysteine (Barefoot and Klaenhammer, 1983; Ridlon et al., 2010). *Lactobacillus acidophilus*, and *L. gasseri* were grown in de Man, Rogosa, and Sharpe broth (MRS, Becton, Dickinson and Company, Sparks, MD, United States), (Barefoot and Klaenhammer, 1983). *Bifidobacterium longum* subsp. *infantis* were grown in MRS supplemented with 500 mg/L L-cysteine (Ventura et al., 2003).

Small Molecule Preparation

2-AI molecules compound 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 (A kind gift of Agile Sciences Inc., Raleigh, NC, United States, **Figure 1**) were provided as a 400 μg/mL stock in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., St. Louis, MO, United States) and were stored at −20°C until use. For all assays, the test compounds were used at a maximum final concentration of 10 μg/mL to ensure efficacy when compared to vancomycin that is currently used for the treatment of CDI (Cohen et al., 2010). Vancomycin (Sigma-Aldrich, St. Louis, MO, United States) was used as a positive control in all assays. Stock solution of vancomycin (8 mg/mL) was diluted in ultrapure water, filter sterilized and stored at 4°C for a week.

Microbroth Dilution for Minimum Inhibitory Concentration Assay

Minimum inhibitory concentration was determined using a modified Clinical and Laboratory Standard Institute (CLSI) broth microdilution method. Test medium used for all *Clostridia* were BHI with 100 mg/L L-cysteine. Bacteroides were grown in Yeast extract casitone fatty acid medium. *Lactobacillus* sp. were grown in MRS. The same medium was supplemented with 500 mg/L L-cysteine for growing *B. infantis*. The inoculum was prepared by the direct colony suspension method. All cell concentrations were adjusted to $\sim 5 \times 10^5$ CFU/mL. An anaerobic environment was maintained at all times using an anaerobic chamber (Coy Industries). An incubation temperature of 37°C was used for all strains. The plates were prepared fresh by making 2-AI molecules or vancomycin dilution stocks in the test media, and adding 90 μL to each well such that the final concentration of the test compounds after the addition of cells (10 μL) ranged from 0.08 to 10 μg/mL. Positive controls included inoculated cells only (in test media to check for media adequacy), and solvent (0.25% DMSO). Uninoculated test media for each strain was used as a negative control to check for sterility. The assay plates were then sealed using a sterile polyester film (VWR, cat # 89134-432) before placing the lid to prevent the panel from dehydrating during incubation. *C. difficile*, *B. fragilis, B. thetaiotaomicron, L. acidophilus, L. gasseri*, and *E. coli* were incubated for 24 h, whereas *C. scindens*, and *C. hylemonae*, were allowed to grow for 48 h. MICs were defined as the lowest concentration at which there was no visible growth. The end point optical density at 600 nm (OD_{600}) of the plates was additionally recorded to measure turbidity.

Growth Kinetics Inhibition Assay

The growth inhibition studies of *C. difficile* were done in a 96-well microtiter plate using previously published methods (Thanissery et al., 2017). All *C. difficile* strains were cultured overnight at 37°C in pre-reduced BHI plus 100 mg/L L-cysteine broth in an anaerobic chamber. Overnight *C. difficile* cultures were sub-cultured 1:10 into same media, and allowed to grow for 3 h anaerobically at 37°C. The culture was then diluted in fresh BHI so that the starting OD_{600} was 0.01. The cell suspension was added in triplicate to a 96-well plate at a final volume of 0.2 ml with the addition of test compound (final concentration: 10 μg/mL), solvent (0.25% DMSO) or vancomycin (final concentration: 2 μg/mL). Each plate contained control wells (without test compounds)

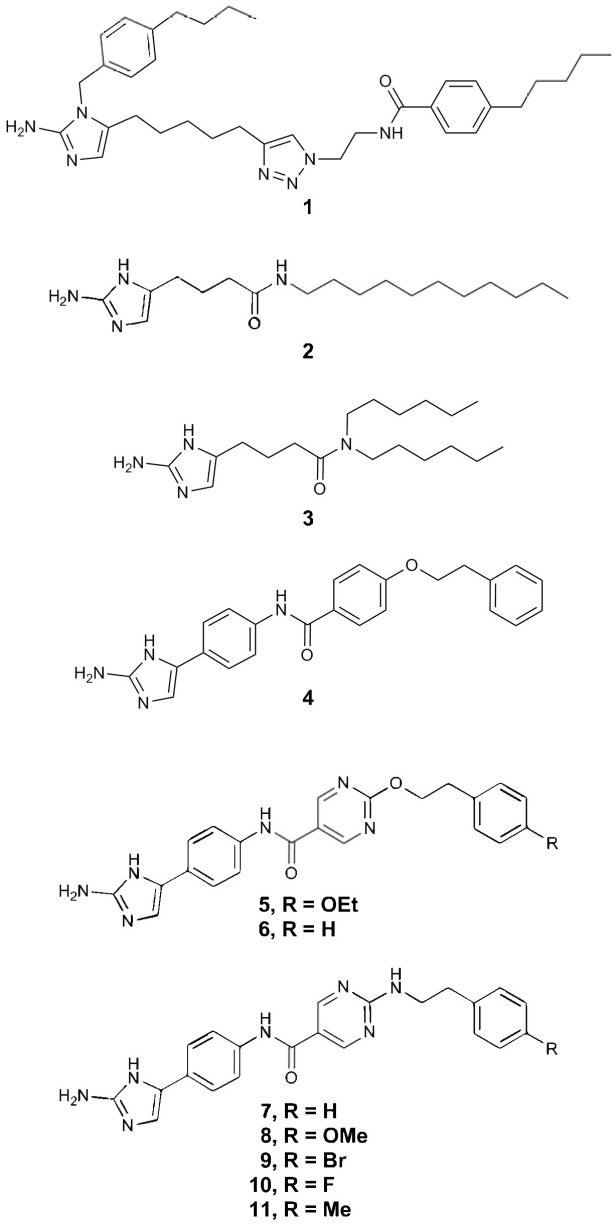

FIGURE 1 | 2-aminoimidazole chemical structures. The chemical structures of all eleven 2-AI molecules are illustrated in this figure.

and blank wells (without cells). The plates were sealed to ensure anaerobic conditions and passed outside the chamber to measure optical density 600 nm (OD_{600}). The optical density was monitored every 30 min for 10 h, shaking the plate for 90 s before each reading, in a Tecan plate reader. A test plate containing 2-AI or vancomycin in media was run before the assay to measure the optical density and ensure the stability of the compounds over the incubation period. After 24 h, the plates were removed from the plate reader and stored in −80°C until use for measuring toxin activity from the culture supernatants.

Toxin Activity Inhibition Assay

Toxin activity was measured by a Vero cell cytotoxicity assay (Winston et al., 2016; Thanissery et al., 2017). Vero cells were grown and maintained in DMEM media (Gibco Laboratories, 11965-092) with 10% fetal bovine serum (Gibco Laboratories, 16140-071) and 1% Penicillin streptomycin solution (Gibco Laboratories, 15070-063). Cells were incubated with 0.25% trypsin (Gibco Laboratories, 25200-056), washed with 1X DMEM media, and harvested by centrifugation 1,000 RPM for 5 min. Cells were plated at 1×10^4 cells per well in a 96-well flat bottom microtiter plate (Corning, 3596) and incubated overnight at 37°C/5% CO_2. Growth inhibition kinetics assay plates were defrosted on ice and then centrifuged at 1,750 RPM for 5 min to pellet vegetative *C. difficile*. Culture supernatants were collected from each well and serially diluted by 10-fold to a maximum of 10^{-6} using 1X PBS. Sample dilutions were incubated 1:1 with PBS (for all dilutions) or antitoxin (performed for 10^{-1} and 10^{-4} dilutions only, TechLabs, T5000) for 40 min at room temperature. Following incubation, these admixtures were added to the Vero cells. After an overnight incubation at 37°C/5% CO_2, plates were viewed under 200× magnification for Vero cell rounding. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in 80% of Vero cells for each sample. Vero cells treated with purified *C. difficile* toxins (A and B) and antitoxin (List Biological Labs, 152C and 155C; TechLabs, T5000) were used as controls. A test cytotoxicity assay was run prior to assays to ensure that the 2-AI molecules did not affect the cytoskeleton of Vero cells at the tested concentrations.

Kill Kinetics Assay

Measurement of OD_{600} Using Plate Reader

Kill kinetics of *C. difficile* were analyzed on a 96-well plate using a modified growth inhibition assay protocol. Briefly, overnight *C. difficile* cultures were back-diluted 1:25 into pre-reduced BHI plus 100 mg/L L-cysteine broth and allowed to grow until it reaches mid log (OD_{600} of 0.45–0.50). The cells were added in triplicates to a 96-well plate at the same volume and concentrations of test compound, solvent, or vancomycin as used in the growth kinetics inhibition assay. Each plate also contained control wells (without test compounds) and blank wells (without cells). The optical density was monitored every 30 min for 12 h, shaking the plate for 90 s before each reading, in a Tecan plate reader.

C. difficile Bacterial Enumeration

Plates were prepared as described here previously for measurement of OD_{600} using a plate reader. Six hours later, 25 μL aliquots were removed from each treatment, serially diluted 10-fold in phosphate buffered saline (PBS), and plated on BHI plus 100 mg/L L-cysteine and 0.1% taurocholate using a track dilution method (Jett et al., 1997). This method involved plating 10 μL of six dilutions on separate tracks of a single square plate (Genesee Scientific, Cat # 26-275). The dilution plate was then heat treated at 65°C for 20 min to kill all vegetative cells. Following heat treatment, the cells were plated on BHI plus 100 mg/L L-cysteine and 0.1% taurocholate. All plates were incubated at 37°C for 24 h anaerobically. Plates were counted the next day to enumerate total vegetative cells plus spores in the unheated samples, and total spores in the heat-treated samples.

Sporulation Inhibition Assay

The sporulation assay is modified from a method previously described as spore inducing and quantification using heat resistance (Shen et al., 2016). Briefly, R20291 spores were streaked on BHI plates containing 100 mg/L L-cysteine plus 0.1% taurocholate and incubated anaerobically for 24 h. The colonies were sub-cultured into 2 mL BHI plus 100 mg/L L-cysteine and were allowed to grow for 4 to 5 h. The turbid culture was centrifuged for 5 min, and the pellet was resuspended in 70:30 broth [per liter contained 63 g Bacto Peptone, 3.5 g Protease Peptone, 0.7 g NH 4 SO 4, 1.6 g Tris Base, 11.1 g BHI Broth, 1.5 g Yeast Extract, supplemented with 3 mL 10% (w/v) Cysteine] to an OD_{600} of ∼0.5. Resuspended cultures (195 μL) with or without test compounds (final concentration: 10 μg/mL), vancomycin (2 μg/mL), or solvent (0.25% DMSO) were incubated at 37°C for 24 h anaerobically. The samples after incubation (20 μL) were serially diluted 10-fold, and 4 μL were plated on BHI plates containing 100 mg/L L-cysteine plus 0.1% taurocholate. The dilution plate was passed out of the chamber for heat treatment at 65°C for 20 min. Four μL from each dilution was plated on BHI plates containing 100 mg/L L-cysteine plus 0.1% taurocholate. All plates were incubated anaerobically at 37°C for 24 h. The number of colony forming units (CFUs) were counted on the lowest dilution in which colonies were visible to determine the CFU/mL of total vegetative cells and spores from the unheated samples and spores only from the heat-treated samples.

Statistical Analysis

Statistical tests were performed using Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla, CA, United States). Significance between treatments and solvent control for toxin activity assay (**Figure 3B**), bacterial enumeration for kill kinetics (**Figure 4**), and sporulation assay (**Figure 5**) were calculated by Student's parametric *t*-test with Welch's correction. Statistical significance was set at a *p*-value of <0.05 for all analyses ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). All assays were done in triplicate.

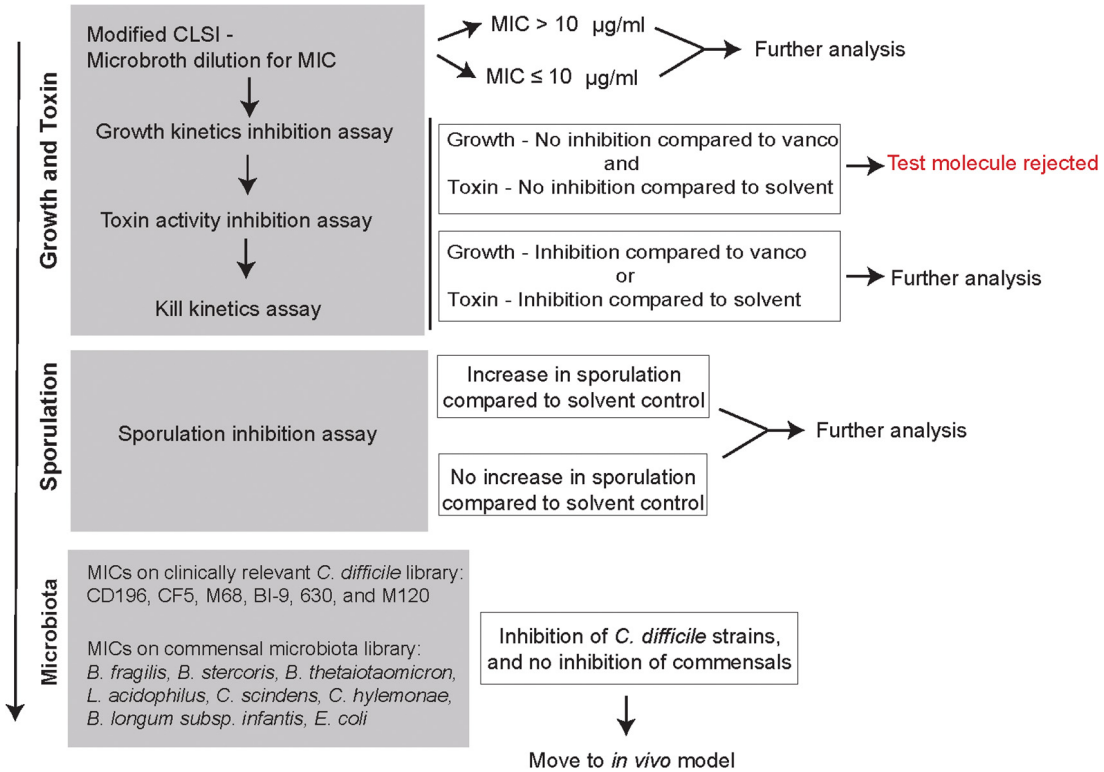

FIGURE 2 | *In vitro* screening pipeline to evaluate 2-aminoimidazole molecules as potential non-antibiotic therapeutics for *C. difficile* infection. Flowchart is designed as an *in vitro* screening pipeline to evaluate small molecules against *C. difficile*. The gray boxes represent the different protocols used to determine how the small molecules affect different stages of *C. difficile* life cycle (growth, toxin, and sporulation) and other members of the gut microbiota. Based on the results, at each box a decision is made whether to advance molecules for further screening with the goal of identifying suitable molecules to be advanced to mouse model testing.

RESULTS

Development of a Screening Pipeline to Test Small Molecule Activity Against Different Stages of the *C. difficile* Life Cycle *in Vitro*

Figure 2 is an overview of the small molecule-screening pipeline that was developed and implemented in this study. The gray boxes represent the different assays that were used to interrogate how small molecules were able to alter different stages of the *C. difficile* life cycle including growth, toxin, and sporulation. We also evaluated their activity against other *C. difficile* strains (CD196, CF5, M68, BI9, 630, M120), and commensals from the gut microbiota (*B. fragilis, B. thetaiotaomicron, L. acidophilus, L. gasseri, C. scindens, C. hylemonae, E. coli*, and *B. longum* subsp. *infantis*) (**Table 1**). All small molecules begin at the first step, screening on a 96-well plate to determine MICs using a microbroth dilution technique. A MIC of 10 µg/ml was considered an initial cut-off for activity when compared to the reference drug vancomycin, which is currently used to treat patients with CDI. This dose was selected because it was hard to sustain concentrations above 10 µg/ml in animal studies based on previous studies with structurally similar compounds. All molecules along with vancomycin (2 µg/ml) and the solvent (0.25% DMSO) were moved down the pipeline, and assayed for growth kinetics inhibition, toxin activity inhibition, and kill kinetics. Molecules that either inhibited growth and or toxin activity were advanced to the next step in the pipeline. A sporulation induction assay was used to determine if the small molecules were able to alter sporulation. All other molecules were moved to the next step in the pipeline where they were screened for activity against other clinical *C. difficile* strains, and a commensal microbiota library. Molecules that show promising antimicrobial or anti-toxin activity sparing the commensals in this pipeline will be further evaluated *in vivo* in a mouse model of CDI.

2-Aminoimidazole Molecules Alter *C. difficile* R20291 Growth and Toxin Activity

The MICs for eleven 2-AI molecules with *C. difficile* are shown in the **Table 2**. Compounds 1, 2, and 3 were the most active against *C. difficile* with MICs ranging from 2.5 to 5 µg/ml. *C. difficile* was not susceptible to all other 2-AI molecules. The control vancomycin had a MIC of 0.15–0.31 µg/ml, and the solvent control (0.25% DMSO) did not inhibit *C. difficile*.

All 2-AI molecules were moved down the pipeline and tested in a *C. difficile* growth kinetics inhibition assay

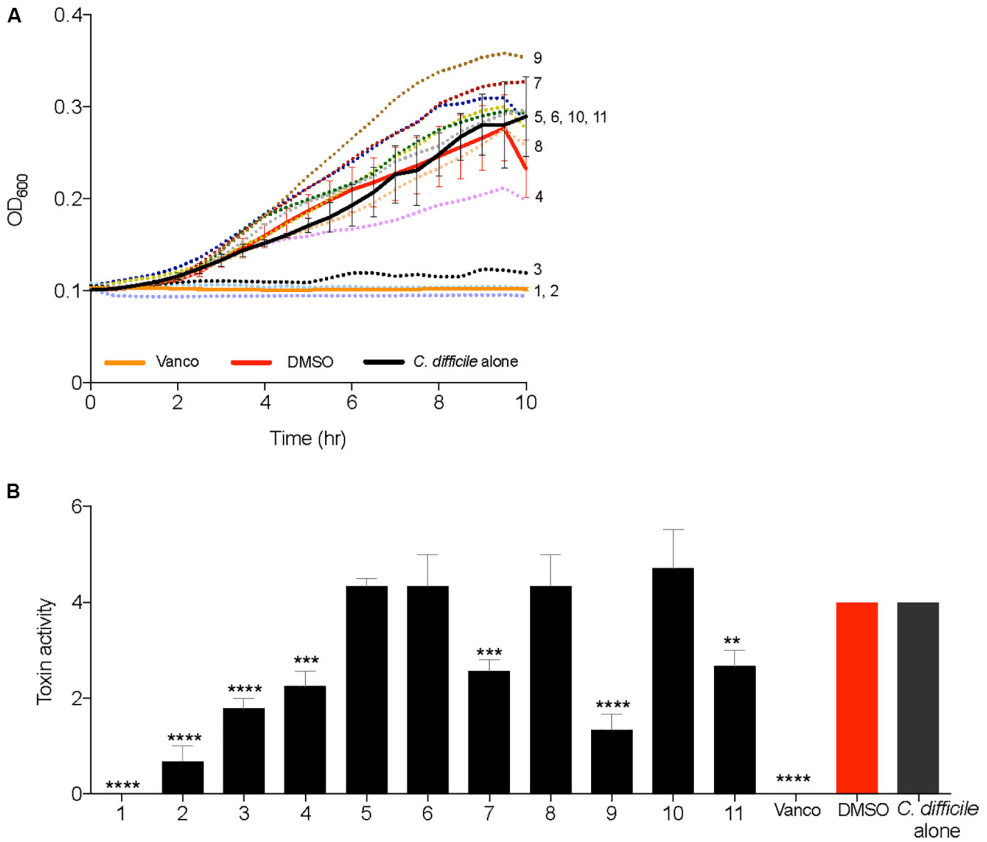

FIGURE 3 | 2-aminoimidazole molecules inhibit growth and toxin activity of *C. difficile*. **(A)** Inhibition of *C. difficile* R20291 growth (OD_{600}) in BHI media supplemented with small molecules (Compound 1 through 11) at a concentration of 10 μg/ml, solvent 0.25% DMSO (DMSO), or 2 μg/ml Vancomycin (Vanco). **(B)** Culture supernatants after 24 h growth inhibition assays were used for Vero cell cytotoxicity assays to measure toxin activity. Toxin titer is expressed as log10 reciprocal dilution toxin per 100 μl of *C. difficile* culture supernatant. Data presented represents mean ± SEM of triplicate experiments. In **(B)** statistical significance between positive control (solvent) and treatment groups was determined by Student's parametric *t*-test with Welch's correction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

at a concentration of 10 μg/ml, along with vancomycin (2 μg/ml), and the solvent (0.25% DMSO). Supplementation of compounds 1, 2, and 3 inhibited the growth of *C. difficile* and was very similar to the vancomycin control (**Figure 3A**). There was no change in *C. difficile* growth kinetics in the presence of all other 2-AI molecules. Toxin activity was measured from culture supernatants of *C. difficile* supplemented with 2-AI molecules in **Figure 3A**. Diminished growth correlated with low toxin activity with the addition of compounds 1, 2, and 3 (**Figure 3B**). Interestingly, growth was unaffected by compounds 4, 7, 9, and 11 yet toxin activity was significantly reduced when compared to the solvent control. The addition of the solvent DMSO to media did not alter *C. difficile* growth or toxin activity. The cytotoxic activity was neutralized at all dilutions containing the sample and antitoxin confirming that the cell rounding was from *C. difficile* toxin. All molecules were advanced to the kill kinetics assay, the next step in the pipeline.

Kill kinetics of *C. difficile* were evaluated by measuring the optical density (OD_{600}) after the addition of 2-AI molecules to cells in mid log growth phase (**Figure 4A**). Supplementation of vancomycin (2 μg/ml) and solvent (0.25% DMSO) were used as controls. Compounds 1, 2, and 3 (10 μg/ml) altered growth, which was further confirmed by enumerating total colony forming units (CFUs) of vegetative cells and spores at the 6 h time point in **Figure 4B**. Addition of 2-AI molecules resulted in a significant log reduction in the total number of vegetative cells and spores for compound 1 (5.5 ± 0.57 log), compound 2 (4.0 ± 0.26 log), and compound 3 (3.6 ± 0.27 log) compared to the solvent control. However, no differences were seen in spores. The solvent DMSO did not affect *C. difficile* kill kinetics like the vancomycin control. 2-AI molecules compound 5, 6, 8, and 10, that did not inhibit growth and toxin activity were rejected at this point in the pipeline. Compounds 1, 2, 3, 4, 7, 9, and 11 were advanced to the next step in the pipeline.

2-Aminoimidazole Molecules Do Not Alter *C. difficile* R20291 Sporulation

Differences in sporulation were determined by inducing spore formation and quantification of heat resistant spores. Sporulation was unaffected when supplemented with DMSO or compounds 1, 2, 3, 4, 7, 9, and 11 (**Figure 5**). All molecules tested for sporulation were advanced to next step of screening in

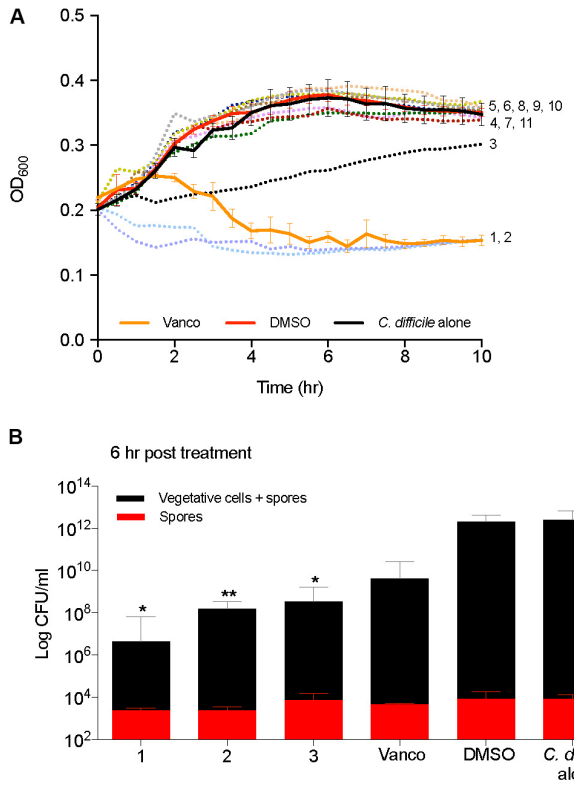

FIGURE 4 | 2-aminoimidazole molecules affect *C. difficile* viability. **(A)** Killing of *C. difficile* R20291 growth (OD_{600}) in BHI media supplemented with small molecules at mid log growth phase (Compounds 1 through 11). **(B)** Total C. *difficile* R20291 vegetative cells and spores, or spores alone 6 h post exposure to small molecules in **(A)** (Compounds 1, 2, and 3) at a concentration of 10 μg/ml when compared to solvent 0.25% DMSO (DMSO, positive control), or 2 μg/ml vancomycin (Vanco, negative control). Data presented represent mean ± SEM of triplicate experiments. Statistical significance between positive control (solvent) and treatment groups was determined by Student's parametric *t*-test with Welch's correction (*$p < 0.05$, **$p < 0.01$).

the pipeline. Spores were also enumerated in the kill assays described previously and no differences were noticed in the spores recovered in the BHI media both at 6 and 24 h post treatment (**Supplementary Figure S1**).

2-Aminoimidazole Molecules Affect Other *C. difficile* Strains Sparing Commensal Members of the Gut Microbiota

Compounds 1, 2, 3, 4, 7, 9, and 11 were screened for MICs against other *C. difficile* strains and a commensal microbiota library. Other *C. difficile* strains (CD196, M68, CF5, 630, BI9, and M120) were inhibited by compounds 1, 2, and 3 at a MIC of 2.5–5 μg/ml and 5–10 μg/ml, respectively (**Table 3**). Vancomycin was inhibitory to all strains at 0.31 μg/ml, except BI9, which was inhibited at 0.16 μg/ml. *C. difficile* strains were not susceptible to all other 2-AI molecules (compounds 4, 7, 9, and 11) at a concentration of 10 μg/ml.

Commensal microbes that are associated with a healthy gut microbiota and colonization resistance against *C. difficile*: *B. fragilis, B. thetaiotaomicron, C. scindens, C. hylemonae* were also susceptible to compound 1 at a MIC of 5–10 μg/ml (**Table 4**). In contrast, all other strains (*L. acidophilus, L. gasseri, E. coli, B. longum* subsp. *infantis*) remained resistant to compound 1 with a MIC greater than 10 μg/ml. Compound 2 was inhibitory to *B. thetaiotaomicron, C. scindens,* and *C. hylemonae* at 10 μg/ml. Interestingly, compound 3, which inhibited *C. difficile* growth, did not have any effect on the commensals at the tested concentration The commensal panel was resistant to compounds 4, 7, 9, and 11 at a concentration of 10 μg/ml, which also did not inhibit *C. difficile* strains.

DISCUSSION

In this study we developed and implemented a small molecule-screening pipeline to screen and select promising compounds that inhibited one or multiple steps in the *C. difficile* life cycle without altering the growth of a panel of gut commensals associated with colonization resistance. 2-AI molecules that have been successfully used to enhance antibiotic activity and mitigate virulence responses against other insidious pathogens were the first compounds screened through our pipeline. We evaluated eleven 2-AI molecules (compound 1 through 11) for their ability to alter *C. difficile* growth, toxin, and sporulation, while sparing other members of the gut microbiota. Compounds 1, 2, and 3 were microbicidal and were able to inhibit and kill *C. difficile* R20291 growth. The antimicrobial activity of compounds 1, 2, and 3 correlated with lower toxin activity. However, there was no difference in the number of spores recovered. Interestingly, compounds 4, 7, 9, and 11 were anti-virulent as they inhibited toxin activity without impacting the growth of *C. difficile* strains and commensals.

Minimum inhibitory concentrations of all molecules were first evaluated with *C. difficile* R20291, and then subsequently moved down the pipeline to evaluate how they affected growth kinetics, and virulence factors such as toxin and sporulation. Treatment with compound 1 (5.5 ± 0.57 log), compound 2 (4.0 ± 0.26 log), and compound 3 (3.6 ± 0.27 log) resulted in a higher log reduction of *C. difficile* vegetative cells and spores then the vancomycin control (2.7 ± 0.50). Based on MIC's, vancomycin (0.15–0.31 μg/ml) was more potent against *C. difficile* R20291 compared to compounds 1, 2, and 3 (2.5–5 μg/ml). Similar sensitivity to vancomycin for *C. difficile* R20291 isolates has been reported (Barbut et al., 2007; Đapa et al., 2013; Brock, 2015). However, different antimicrobial sensitivity testing methods were used making it difficult to compare between studies. Since vancomycin is bacteriostatic to logarithmic phase cultures, it was not surprising that there was a lower log reduction at 2 μg/ml (Levett, 1991; Alam et al., 2015). Several antimicrobials with a range of modes of action are under clinical evaluation for CDI now (Kociolek and Gerding, 2016). Surotomycin is a novel lipopeptide that has antibacterial

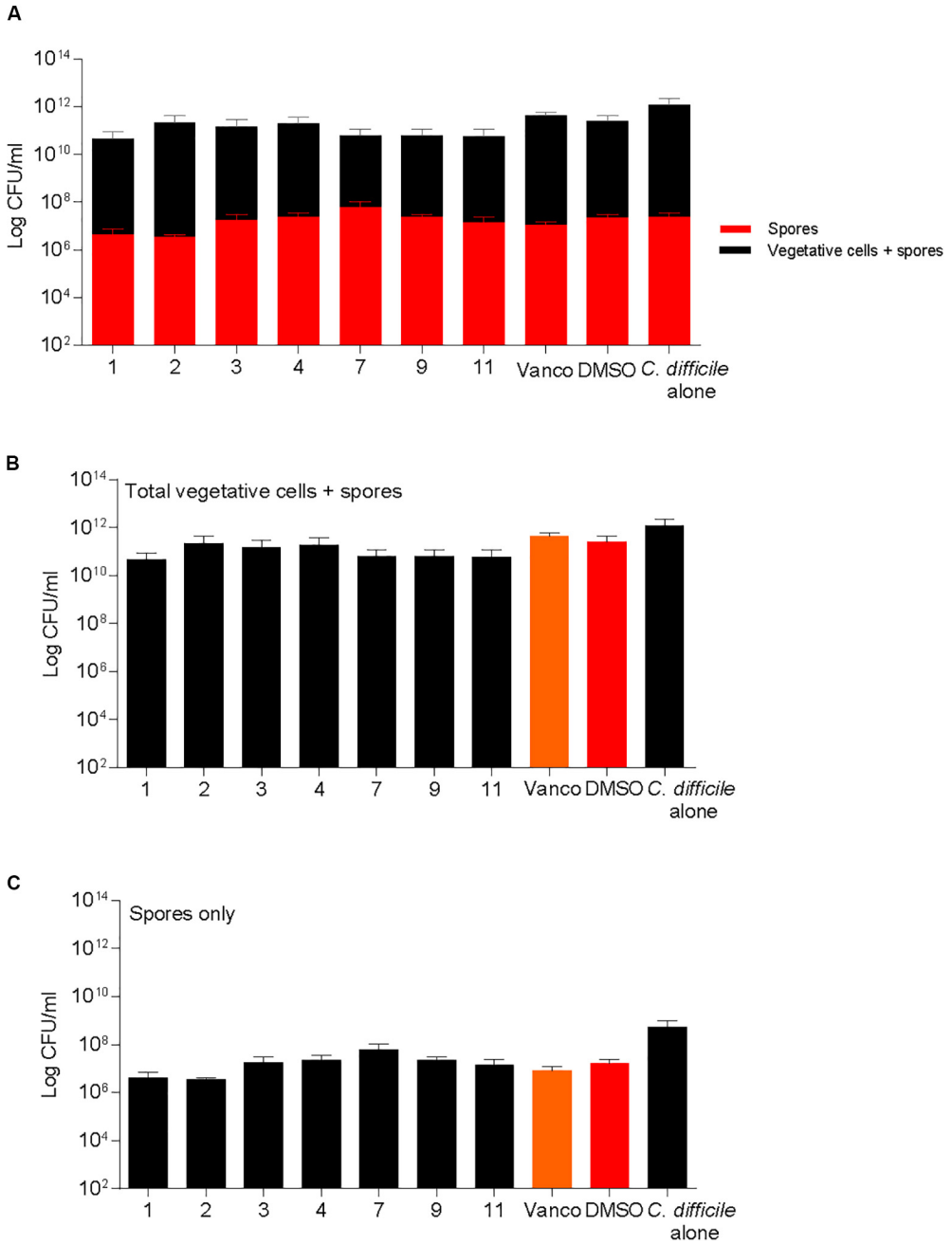

FIGURE 5 | 2-aminoimidazole molecules do not alter *C. difficile* sporulation. Sporulation of *C. difficile* on 70:30 agar plates after supplementation with 2-AI molecules (Compounds 1, 2, 3, 4, 7, 9, and 11) at a concentration of 10 μg/ml when compared to solvent 0.25% DMSO (DMSO, positive control), or 2 μg/ml vancomycin (Vanco, negative control) after 24 h. Data represents **(A)** Total vegetative cells and spores, and spores only, **(B)** Total vegetative cells and spores, **(C)** Spores only at 24 h post treatment.

activity by disrupting the bacterial cell membrane (Mascio et al., 2012). It has potent activity against *C. difficile* and reduced activity against commensal bacteria (Citron et al., 2012). However, it was not associated with lower recurrence rates in phase III clinical trials (Boix et al., 2017). Cadazolid is another novel oxazolidinone compound which inhibits protein synthesis (Locher et al., 2014a). This compound reduces toxin production and sporulation *in vitro* in the absence of bacterial killing (Locher et al., 2014b). Ridinilazole a DNA synthesis inhibitor is a novel narrow spectrum antibiotic and has shown promising phase II results (Basseres et al., 2016; Steinebrunner et al., 2018). The mode of action for compounds 1, 2, and 3 screened in our study is unknown, and more studies are needed to explore bactericidal targets

TABLE 3 | Minimum inhibitory concentration of 2-aminoimidazole molecules against other *C. difficile* strains compared to vancomycin.

***C. difficile* strain**	**MIC (μg/mL)**			
	Compound 1	**Compound 2**	**Compound 3**	**Vancomycin**
CD196	5	5	5	0.31
M68	5	10	10	0.31
CF5	5	5	10	0.31
630	5	5	5	0.31
BI9	2.5–5	5	5	0.16
M120	5	10	10	0.31

Minimum inhibitory concentration (MIC) was determined by broth microdilution as per modified CLSI guidelines for anaerobes. Data represent mean values from triplicate trials.

TABLE 4 | Minimum inhibitory concentration of 2-aminoimidazole molecules on commensal microbiota library.

Commensal strain	**MIC (μg/mL)**			
	Compound 1	**Compound 2**	**Compound 3**	**Vancomycin**
B. fragilis	10	>10	>10	2.5
B. thetaiotaomicron	5	10	>10	1.25
L. acidophilus	>10	>10	>10	0.31
L. gasseri	>10	>10	>10	0.16
C. scindens	5–10	10	>10	0.31
C. hylemonae	10	10	>10	1.25–0.31
E. coli	>10	>10	>10	>10
B. longum subsp. *infantis*	>10	>10	>10	0.63

Minimum inhibitory concentration (MIC) was determined by broth microdilution as per modified CLSI guidelines for anaerobes. Data represent mean values from triplicate trials.

including cell wall biosynthesis, DNA replication, and protein synthesis.

Targeting virulence is a therapeutic approach that provides promising opportunities to inhibit pathogenesis *in vivo* without affecting bacterial growth (Cegelski et al., 2008). Mitigating virulence shifts the advantage to the host since the immune response remains unimpaired by the bacteria. Additionally, the gut microbiota that provide colonization resistance against *C. difficile* are unaltered, reducing recurrence. Common anti-toxin agents pursued as potential therapeutics for various infectious diseases include inhibitors of toxin transcription factors (Hung et al., 2005), toxin trafficking molecules (Saenz et al., 2007), and the use of toxin neutralizing antibodies (Arnon et al., 2006). Quorum sensing molecules (Hentzer et al., 2003) and bacterial two-component response systems that are central to bacterial virulence are often targeted for anti-virulence effect as well. In our study, compounds 4, 7, 9, and 11 did not affect growth, yet toxin activity decreased significantly compared to the solvent control. This is in line with the mechanism of action of 2-AI molecules that are able to target response regulator protein of bacterial TCS, thereby inhibiting virulence determinants such as antibiotic resistance, toxin secretion, and biofilm formation in other antibiotic resistant bacteria including *P. aeruginosa, A. baumannii,* and *S. aureus* (Rogers et al., 2010; Brackett et al., 2014; Draughn et al., 2017). In *C. difficile*, TCS is a part of the quorum sensing system called accessory gene regulator (agr) system that regulates toxin synthesis (Darkoh et al., 2015). The components of the agr system in strain R20291 includes *agrB1* and *agrD1* within the *agr1* loci, that are responsible for producing the quorum signaling autoinducer peptide, and *agrB2D2* and *agrC2A2* within *agr2* loci that are quorum signal-generation and response genes, respectively (Darkoh et al., 2016). *C. difficile* also has a Spo0A histidine kinase TCS system that is known to play a key role in both sporulation and toxin production (Underwood et al., 2009). However, the molecular mechanisms that lead to the control of toxin production by Spo0A are found to be strain dependent, and are not well characterized (Darkoh et al., 2015; Martin-Verstraete et al., 2016). Inhibition of any components in the accessory gene regulator pathway, and Spo0A histidine kinase TCS system could result in significant control of the toxin.

Targeting the toxin protein itself rather than bacterial growth to treat CDI is gaining momentum especially after *tcdA* and *tcdB* knockouts of toxigenic *C. difficile* proved to be avirulent in a hamster model (Kuehne et al., 2014). Both toxins are composed of four large domains: putative receptor binding domain, a transmembrane domain, a CPD, and a glucosyltransferase domain, whose conformational changes and the subsequent events leads to cytopathic and cytotoxic effect of the toxins (Pruitt and Lacy, 2012). These domains are potential drug targets for toxin inactivation. Bezlotoxumab an injectable human monoclonal antibody was FDA approved recently for the prevention of recurrent CDI. The antibodies bind to the receptor binding domain of toxin B when given systemically, thereby

mitigating the *in vivo* effects of the toxin (Yang et al., 2015; Wilcox et al., 2017). A viable alternate strategy to target toxins is by using small molecules that could be delivered directly to the site of infection rather than systemic administration. Indeed, a promising bioactive compound, ebselen, which is currently under clinical investigation for unrelated indication was found to inhibit CPD activity *in vitro*. Ebselen was also validated in a mouse model to bind toxin B, and thereby prevent *C. difficile* induced clinical pathology (Bender et al., 2015). In another study using a chemical genetics strategy, several small molecules were screened to target potential domains and pathways. This study laid the foundation for identifying first-generation inhibitors of toxin B that mediate CDI (Tam et al., 2015). Antitoxin molecules represent a novel paradigm and could provide the industry with new opportunities in the treatment and management of CDI.

Since 2-AI molecules could potentially affect Spo0A histidine kinase TCS system that controls sporulation, we attempted to measure the inhibitory activity of 2-AI molecules on sporulation induction of mid log *C. difficile* cells. No differences were observed in the number of spores recovered with or without the addition of 2-AI molecules at a concentration of 10 μg/mL. Compounds 1, 2, and 3 were growth inhibitory at this concentration, however, it is crucial to evaluate if the 2-AI molecules induce stress on the cells resulting in increased spore formation. Fidaxomicin is the only drug currently available that inhibits sporulation when sub inhibitory concentrations are added to early stationary phase cells (Babakhani et al., 2012). Anti-sporulation properties would provide greater effectiveness to control transmission and reduce recurrences of CDI.

Since the gut microbiota plays a key role in providing colonization resistance against *C. difficile* (Theriot et al., 2014; Buffie et al., 2015), we tested the small molecules against eight different bacterial strains that are members of the healthy human gut microbiota, and six other *C. difficile* strains from distinct PCR ribotypes. We included members from four of the five dominant phyla of the gut microbiota including Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Tremaroli and Backhed, 2012). Firmicutes make up 50–70% of the colonic bacterial community (Frank and Pace, 2008). Members of Firmicutes including *L. acidophilus, L. gasseri, C. scindens, C. hylemonae, C. heranonis* were added to the panel. *B. fragilis* and *B. sterocis* belonging to the phyla Bacteroidetes were added to the panel as they are designated as key stone species in the human gut microbiome (Fisher and Mehta, 2014). Another member of Bacteroidetes added was *B. thetaiotaomicron*. This commensal is found to antagonize intestinal pathogens through a range of mechanisms (de Sablet et al., 2009; Ferreira et al., 2011; Kamada et al., 2012). *B. infantis* belonging to the phyla Actinobacteria known to synthesize compounds necessary for functional maturation of enterocytes and host immunity, were also added to the panel (Round and Mazmanian, 2009; Guinane et al., 2011). Compounds 4, 7, 9, and 11 used at a concentration that inhibited *C. difficile* toxin activity had no effect on the commensal panel. Compounds 1 and 2 were microbicidal to *C. difficile*, but remained resistant to most of the commensal panel except for Bacteroides and the commensal *Clostridia*. Compound 3 had narrow spectrum activity against *C. difficile,* and did not affect growth of the commensal microbiota at a concentration of 10 μg/ml.

Screening novel small molecules against *C. difficile* rely on MIC assays or growth inhibition assays by measuring optical density in a plate reader. This is not always an accurate readout as exposure of *C. difficile* to stressors is able to increase sporulation (Wilcox and Fawley, 2000; Fawley et al., 2007). A drop in optical density overtime in a growth inhibition assay does not distinguish between vegetative cell lysis and spore formation. It is also important to evaluate viable counts of vegetative cells and spores to confirm true growth inhibition. In this study, growth was evaluated in multiple assays including a growth kinetics inhibition assay (microbroth dilution technique and OD_{600} measurement on cells in early log phase), and a kill kinetics assay (OD_{600} measurement and bacterial enumeration of cells in mid log phase). Another strength of our pipeline is that it takes into consideration other *C. difficile* strains from distinct ribotypes to ensure there are no differences in susceptibility across strains. Additionally, understanding how these compounds affect other gut commensal bacteria is important for the restoration of colonization resistance *in vivo*. The pipeline not only allows for quick screening of antimicrobials, but also for anti-virulence agents. The test molecule concentrations selected for screening can be modified based on each molecule.

There are many strengths to using this small-molecule screening pipeline, however, there are some limitations. We did not evaluate the first stage of the *C. difficile* life cycle, spore germination. However, addition of this assay to the pipeline in the future could be valuable. Although the Vero cell cytotoxicity assay we use in this study is the gold standard for evaluating toxin activity it is semi-quantitative, and other assays such as qRT-PCR and immunoblotting are more quantitative. Another limitation of our toxin assay is that the BHI media used for culturing was supplemented with cysteine, which can reduce toxin expression (Karlsson et al., 2000; Dubois et al., 2016). However, controls using the same media were used for comparison which ensures equal impact across all treatments. The sporulation assay also has limitations as it evaluates sporulation induction when test molecules are added ≥MICs and incubated for 24 h. Therefore, the results of the sporulation assay were not used as a criterion to move the test molecules to the next level of screening. Further testing evaluating sporulation inhibition could be done by adding sub-inhibitory concentrations of test molecules to *C. difficile* cultures and allowing an extended period of incubation before spore enumeration.

Finally, future studies are needed to characterize the anti-toxin activity and understand the mode of action for these 2-AI compounds. The next step after completing the pipeline is to test the therapeutic properties of 2-AI molecules in a mouse model. Etiology of CDI is complex and a combined approach of drugs inhibiting different stages of *C. difficile* life cycle are advantageous for the treatment and management of CDI.

AUTHOR CONTRIBUTIONS

RT, DZ, RD, and CT conceived and designed the experiments. RT performed the experiments. RT and CT performed the analysis. RT, DZ, RD, and CT wrote and edited the manuscript.

ACKNOWLEDGMENTS

We thank the following researchers for providing us with *C. difficile* and commensal strains used in this study: Trevor Lawley, Joe Sorg, Aimee Shen, Eric Martens, Jason Ridlon, and Rodolphe Barrangou. We also thank David Jung for synthesizing the 2-AI compounds tested.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01206/full#supplementary-material

FIGURE S1 | 2-aminoimidazole molecules do not alter *C. difficile* spores recovered in BHI media at 6 and 24 h post treatment. **(A)** Total C. *difficile* R20291 vegetative cells and spores, **(B)** total vegetative cells and spores, **(C)** spores only at 6 h and **(D)** total C. *difficile* R20291 vegetative cells and spores, **(E)** total vegetative cells and spores, **(F)** spores only at 24 h for Compounds 1, 2, and 3 at a concentration of 10 μg/ml when compared to solvent 0.25% DMSO (DMSO, positive control), or 2 μg/ml vancomycin (Vanco, negative control). Data presented represent mean ± SEM of triplicate experiments. Statistical significance between positive control (solvent) and treatment groups was determined by Student's parametric *t*-test with Welch's correction (*$p < 0.05$, **$p < 0.01$).

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Conflict of Interest Statement: CT is a scientific advisor to Locus Biosciences, a company engaged in the development of antimicrobial technologies. DZ and RD are employees of Agile Sciences, Inc., a company engaged in the development of antimicrobial technologies.

The other author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 23 May 2018
doi: 10.3389/fmicb.2018.01009

Targeting *Clostridium difficile* Surface Components to Develop Immunotherapeutic Strategies Against *Clostridium difficile* Infection

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New therapies are needed to prevent and treat *Clostridium difficile* infection and to limit the rise in antibiotic resistance. Besides toxins, several surface components have been characterized as colonization factors and have been shown as immunogenic. This review will focus on passive and active immunization strategies targeting *C. difficile* surface components to combat *C. difficile*. Concerning passive immunization, the first strategies used antisera raised against the entire bacterium to prevent infection in the hamster model. Then, surface components such as the flagellin and the S-layer proteins were used for immunization and the passive transfer of antibodies was protective in animal models. Passive immunotherapy with polyvalent immunoglobulins was used in humans and bovine immunoglobulin concentrates were evaluated in clinical trials. Concerning active immunization, vaccine assays targeting surface components were tested mainly in animal models, mouse models of colonization and hamster models of infection. Bacterial extracts, spore proteins and surface components of vegetative cells such as cell wall proteins, flagellar proteins, and polysaccharides were used as vaccine targets. Vaccine assays were performed by parenteral and mucosal routes of immunization. Both gave promising results and pave the way to development of new vaccines.

Keywords: *Clostridium difficile*, surface components, passive immunotherapies, vaccines, protection

OPEN ACCESS

Edited by:
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Reviewed by:
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The University of Nottingham, United Kingdom
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *01 March 2018*
Accepted: *30 April 2018*
Published: *23 May 2018*

Citation:
Péchiné S, Bruxelle JF, Janoir C and Collignon A (2018) Targeting Clostridium difficile Surface Components to Develop Immunotherapeutic Strategies Against Clostridium difficile Infection. Front. Microbiol. 9:1009. doi: 10.3389/fmicb.2018.01009

INTRODUCTION

Clostridium difficile is involved in a wide spectrum of diseases from mild post-antibiotic diarrhea to pseudomembranous colitis (PMC) that can be severe and lead to complications such as toxic megacolon, septic shock, and death (Rupnik et al., 2009). *C. difficile* infections (CDI) are often nosocomial but can also be community-acquired. Contamination occurs with ingestion of spores, which germinate in the intestinal tract allowing vegetative cells to colonize the gut and multiply in the colon. Several studies showed that several surface components have a role in the colonization step. Colonization is favored by dysbiosis of the intestinal microbiota often due to antibiotic treatment. Then, the toxins TcdA and TcdB are released and are responsible for the intestinal symptoms. The treatment of a first episode is based on specific antibiotic treatment. However, a major problem of these infections is the high level of recurrences 20–30% after a first treatment of an initial infection and more than 50% after a first recurrence (Shields et al., 2015). CDI is an important healthcare-associated disease, causing almost 500,000 infections each year in the United States. *C. difficile* infections have a significant cost on healthcare systems (Lessa et al., 2015). The United States Center for Disease Control has designated this pathogen as the pre-eminent of

three "Urgent Threats" to United States healthcare. Besides first line antibiotic treatments, which target *C. difficile* but also species of the normal microbiota, new therapeutic strategies are needed to treat CDI and prevent recurrences. These new strategies must preserve the normal intestinal microbiota and its barrier effect and limit rise in antibiotic resistance.

A better knowledge of *C. difficile*-host interaction has highlighted the numerous risk factors of CDI. Susceptibility to CDI is well-known and depends on host exposure to *C. difficile*, treatments facilitating *C. difficile* host colonization and a wide range of host characteristics. Host risk factors are age greater than 65 years, co-morbidities such as chronic diseases and host immune response. Several studies have reviewed the role of innate and adaptive immunity in CDI outcome (Madan and Petri, 2012; Péchiné and Collignon, 2016; Rees and Steiner, 2017; Vargas et al., 2017). For over 30 years, immunodeficiency and hematological malignancies such as neutropenia have been described as important risk factors for severe CDI (Rampling et al., 1985; Selvey et al., 2016). In the United States, between 2005 and 2011, a study on more than one million patients with leukemia showed that the susceptibility to CDI in this population was 2.6 times greater than non-leukemia patients (Luo et al., 2015). However, in humans it is difficult to pinpoint the role of the immune response in the susceptibility to CDI because of the accumulation of risk factors in immunodeficient patients such as multiple antibiotic treatments (Fisher et al., 2014).

Interestingly, several studies showed that the intensity of the specific host immune response to *C. difficile* exposure is a key factor in CDI outcome. Thus, the inability to develop a humoral immune response to *C. difficile* may be a prediction marker for severe and/or recurrent CDI (Kelly, 2012). In particular, anti-toxin antibody levels have been correlated with the severity, duration and recurrence rate of CDI (Warny et al., 1994; Kyne et al., 2000, 2001; Leav et al., 2010; Wullt et al., 2012). The first developed immunotherapies targeted toxins with the promising but recently stopped clinical development of CDIFFENSE vaccine by Sanofi Pasteur and with the FDA approved passive immunotherapy targeting TcdB, bezlotoxumab (Navalkele and Chopra, 2018). Although promising results in the decrease of CDI and recurrences, vaccines and passive immunotherapies targeting toxins do not appear to prevent *C. difficile* gut colonization, and further dissemination of spores in the environment. Therefore, another approach to prevent CDI and recurrences is to directly target the bacterium by developing immunotherapeutic strategies specific for *C. difficile* surface components.

In this review, first we introduce the characterized *C. difficile* surface components and the host specific immune response, then we evaluate the different passive and active immunization strategies targeting *C. difficile* surface components.

HOST IMMUNE RESPONSE AGAINST *C. difficile* SURFACE COMPONENTS

Serum antibodies against *C. difficile* surface components have been found in patients with CDI (Pantosti et al., 1989; Mulligan et al., 1993). Pantosti et al. (1989) compared the antibody response in the acute and convalescent phase and observed that the IgG response increased during disease outcome. Mulligan et al. (1993) found that the level of immunoglobulins against somatic cell antigens was higher in asymptomatic than in symptomatic carriers.

A better knowledge of *C. difficile* pathogenesis has shed light on several components involved in intestinal colonization.

Surface Proteins of the Vegetative Cells

A major family of surface proteins is the cell wall protein (CWP) family (Willing et al., 2015), in which the S-layer proteins, the adhesin Cwp66, and the protease Cwp84 are implicated in the colonization step (Janoir, 2016).

The two S-layer proteins (SLPs) are the most abundant surface proteins and form a two-dimensional array around the bacterium. They derive from the S-layer precursor SlpA after cleavage by the Cwp84 protease (Kirby et al., 2009; Dang et al., 2010). The low-molecular-weight (LMW)-SLP is surface-exposed and highly variable between strains while the high-molecular-weight (HMW)-SLP is conserved between strains and anchored in the cell wall (Karjalainen et al., 2001). Both SLPs are capable of adhering *in vitro* to human gastro-intestinal tissue and intestinal epithelial cells (Calabi et al., 2002; Merrigan et al., 2013). The HMW-SLP also adheres to extra cellular matrix (ECM) proteins including type 1 collagen, thrombospondin and vitronectin (Calabi et al., 2002). In addition, SLPs interact with TLR 4 inducing a pro-inflammatory innate immune response of the host (Ryan et al., 2011). Then, SLPs induce an adaptive immune response. For a long time, it has been impossible to obtain SLP mutants, suggesting essentiality of the *slpA* gene. Recently, Kirk et al. (2017) described rare resistant mutants to a diffocin (Av-CD 291.2), which selectively kills *C. difficile* strains. Two of these resistant mutants displayed a SLP-null phenotype. These mutants displayed severe sporulation defects, and were capable of colonizing the intestinal tract of hamsters despite a complete attenuation of virulence in this animal model (Kirk et al., 2017).

Wright et al. (2008) by an immuno-proteomic-based approach demonstrated that the SLPs are detected by patient sera suggesting that these proteins are immunodominant and expressed during infection (Wright et al., 2008). Drudy et al. (2004) found that antibody response to SLPs (total extracts of both SLPs) was not significantly different between patients with symptomatic CDI, asymptomatic *C. difficile* carriers and healthy controls. However, "patients with recurrences compared to patients with a single episode of CDI did not mount a SLP-specific IgM immune response on days 3, 6, and 9 after onset of diarrhea" (Drudy et al., 2004). Recently, Bruxelle et al. assessed the immune response of the host against the SlpA precursor. In humans SlpA was immunogenic and patients with CDI had a significantly higher level of anti-SlpA antibodies as compared to healthy patients (Bruxelle et al., 2016). Interestingly, Negm et al. (2015) developed a microarray assay to screen antibody responses simultaneously against various *C. difficile* antigens from different *C. difficile* strains. By this assay the authors tested IgG antibodies against native or recombinant toxins and SLP extracts from various *C. difficile* strains in a CDI patient group, a group of patients with cystic fibrosis and a healthy

group of subjects. Patients with CDI had significantly lower anti-SLP IgG levels with all strains tested (ribotypes 001, 002, 027) compared to the two other groups. These different results could be explained by the diversity of subjects in CDI groups but also in healthy control groups for which previous exposure to *C. difficile* in the community may lead to production of specific anti-SLP antibodies. In addition, variation in these data could also be explained by differences in the methodologies for antibody characterization, especially regarding the antigens, SLP- extracts, LMW-SLP, HMW-SLP or the precursor SlpA.

The Cwp66 protein also displays adhesin properties and its expression is increased *in vitro* after heat-shock (Waligora et al., 2001). It has a two-domain structure with the C-terminal domain (C-ter) surface exposed and highly variable between strains whereas the N-terminal domain is conserved and anchored in the call wall.

The protease Cwp84 is surface exposed and conserved between strains (Janoir et al., 2007; Chapeton Montes et al., 2013). Besides cleavage of the SlpA precursor into the two mature SLPs, this protease is capable of degrading *in vitro* collagen, fibronectin or vitronectin. These proteolytic properties may *in vivo* facilitate bacterial spread (Janoir et al., 2007). However, a *cwp84* mutant is as virulent as the wild type strain in the hamster model suggesting that this protease has a limited function in the pathogenesis and can be substituted by another protease such as Cwp13 (Kirby et al., 2009).

The C-terminal domain of Cwp66 and the Cwp84 protease are immunogenic in humans. Péchiné et al. comparing total antibody levels in a CDI group and a control group observed that the mean level of antibodies against Cwp66 C-ter and Cwp84 was statistically lower in the CDI group. These results suggest that the antibodies can be protective against CDI (Péchiné et al., 2005a,b).

Other proteins are surface-exposed and implicated in the colonization step. The heat shock protein GroEL is released extracellularly after heat shock and capable of binding to epithelial cells (Hennequin et al., 2001). This protein could serve adhesin function in case of various *in vivo* shocks. After immunization of hamsters with a non-toxigenic *C. difficile* strain, GroEL has been identified by an immuno-proteomic approach as a key factor leading to production of protective antibodies (Péchiné et al., 2013). GroEL is highly conserved between *C. difficile* strains but also displays high homology with other bacterial species of the normal microbiota. Thus, these homologies limit the interest of GroEL as vaccine antigen. The lipoprotein CD630-0873 displays adhesive properties on Caco-2 cells. In addition, a knockout mutant adheres significantly less than the parental strain to Caco-2 cells confirming the role of the lipoprotein in adherence (Kovacs-Simon et al., 2014).

The fibronectin-binding protein Fbp68 binds to fibronectin (soluble and immobilized) and to intestinal fibronectin-pre-incubated cells. Fbp68 could interact with fibronectin in host tissues. It is highly conserved between strains and displays 38 and 39% identity, respectively with PavA of *Streptococcus pneumoniae* and Fbp54 of *Streptococcus pyogenes* (Hennequin et al., 2003; Barketi-Klai et al., 2011). Manganese binds to the N-terminal domain of Fbp68 inducing a change of conformation of the protein essential for fibronectin binding (Lin et al., 2011). Interestingly knockout mutants of the *fbp* gene adhered more *in vitro* to epithelial cells than the parental strain confirming that adherence is a multifactorial process. *In vivo*, in monoxenic mice caecal colonization by the mutant was reduced compared to the wild type and in dixenic mice its intestinal implantation was slower but caecal colonization was similar (Barketi-Klai et al., 2011). So the adhesin Fbp68 could participate to intestinal colonization. This protein is expressed during the course of infection and is immunogenic in humans (Péchiné et al., 2005a). The collagen-binding protein CbpA has an N-terminal collagen-binding domain and a C-terminal domain anchored to the cell wall. A recombinant protein expressing the N-terminal domain was able to bind to collagen *in vitro* and expression of CbpA in *Lactococcus lactis* led to enhanced adherence to collagen (Tulli et al., 2013). However, a *cbpA* knockout strain adhered similarly to collagen compared to the wild type isogenic strain. Similarly, in a dixenic mouse model of colonization, the mutant and the parental strain showed similar level of intestinal colonization (Janoir et al., 2013). CbpA by interacting with host ECM proteins after disruption of the tight junctions by the toxins could facilitate the pathogenic process. Two teams characterized a secreted-zinc metalloprotease ZmP1 (CD630-28300) capable of cleaving IgA2, fibrinogen or fibronectin (Cafardi et al., 2013; Hensbergen et al., 2014). According to c-di-GMP concentration (low concentration), this protease is produced and can limit adherence to host proteins.

Surface Filamentous Structures: Flagella and Pili

Most *C. difficile* strains are motile, however in some strains the flagellar organization is peritrichous, and in others polar. In addition, a few strains such as the PCR-ribotype 078 strains are not motile since the F3 flagellar locus is absent. The flagellin FliC and the cap protein FliD bind *in vitro* to murine mucus. *In vivo*, it was shown that non-flagellated strains adhered significantly less than flagellated strains to mouse caeca. These first results are in favor of the role of flagella in intestinal colonization (Janoir, 2016).

More recently, other authors compared the adherence and colonization properties of parental motile strains to knockout *fliC* or *fliD* mutants (Baban et al., 2013). Surprisingly, the results were different between 630 strain and R20291 027 strain. In the 630 background, *fliC* and *fliD* mutants displayed increased adherence to intestinal epithelial cells compared to the parental strain (Baban et al., 2013). In contrast, in the R20291 background *fliC* and *fliD* mutants showed decreased adherence compared to the parental strain. These authors concluded that for "the 630 strain flagella and motility are not essential for adherence and colonization whereas for *C. difficile* R20291 flagella may play a more significant role in bacterial adherence than initial motility-driven colonization." Taken together these results showed that the role of flagella in *C. difficile* pathogenesis is complex and different according to strains. In addition, flagella can modulate toxin production and stimulate innate immunity via flagellin-TLR5 interaction. *C. difficile* flagella and flagellin have been shown to interact with TLR5 in various human epithelial cells

(Yoshino et al., 2013; Batah et al., 2016). This interaction leads to CCL20 and IL-8 production. In an *in vivo* mouse model, Batah et al. (2016) compared cecal inflammation after challenge with the R20291 027 wild type strain or with various flagellar and toxin mutants. Their results strongly suggest that the flagellin in combination with toxins is responsible for intestinal inflammation.

After stimulating innate immunity flagella also induce an adaptive immune response. Patients with CDI develop antibodies to FliC and FliD. Péchiné et al. (2005a) showed that the mean level of serum antibodies against FliC and FliD was statistically lower in a CDI patient group as compared to a control group. These results concerning flagellar proteins but also CWPs and Fbp68 suggest a protective role of antibodies targeting colonization factors.

Genes for type IV pili are present in *C. difficile* strains such as the 630 and 027 R20291 strains. Nine pilin or pilin-like protein genes were described in the *C. difficile* R20291 genome. The N-terminal hydrophobic regions of these pilins are relatively conserved but their C-termini are divergent (Maldarelli et al., 2014; Piepenbrink et al., 2015). This genetic variability probably facilitates immune evasion to the host immune response. In *C. difficile*, Type IV pili have been shown to be involved in bacterial auto-aggregation at high c-di-GMP concentrations (Bordeleau et al., 2015).

Polysaccharides and Lipoteichoic Acid

Ganeshapillai et al. (2008) identified PS-I and PS-II, two *C. difficile* cell wall polysaccharides. PS-I is a polymer of branched pentasaccharide phosphate-repeats composed of rhamnose and glucose. PS-II is a polymer of hexasacharide phosphate-repeats composed of glucose, mannose, *N*-aceylgalactosamine. PS-II appears to be a common antigen of *C. difficile* conserved across the majority of *C. difficile* strains.

PS-I is expressed in low levels in bacterial cultures and consequently is difficult to obtain by extraction. For this reason, Martin et al. (2013) synthesized the pentasaccharide repeating unit of PS-I and oligosaccharide substructures using boosted chemical synthesis protocols and produced large amounts of well-defined PS-I related glycans. The immunogenicity of this PS-I glycan has been confirmed by specific humoral immune response analysis in stool and serum samples obtained from CDI patients.

PSII is the most abundant polysaccharide expressed by most *C. difficile* ribotypes. Danieli et al. (2011) synthesized the surface polysaccharide PS-II repeating unit. To be fully immunogenic, polysaccharides have to be coupled to carrier proteins. Adamo et al. (2012) explored a combination of chemical synthesis to identify a synthetic fragment that after conjugation to a carrier protein would be immunogenic. They demonstrated that the phosphate group was a key factor in synthetic glycans to mimic the native PSII polysaccharide. Both native PSII and a phosphorylated synthetic hexasaccharide repeating unit conjugated to the diphtheria toxoid variant CRM197 could elicit immunogenic responses in mice (Adamo et al., 2012). The synthetic PS-II hexasaccharide hapten was specifically recognized by IgA antibodies extracted from stools of patients with CDI. Oberli et al. (2011) used PS-II combined with CRM197 to immunize mice by subcutaneous route. The PS-II-CRM197 conjugate was immunogenic and induced specific IgG antibodies in the serum of immunized mice (Oberli et al., 2011). In another study, a non-adjuvanted PSI/PSII preparation was administered to pregnant sows as vaccine antigens and IgM antibodies specific for PSII were then elicited (Bertolo et al., 2012). Romano et al. (2014) evaluated in a mouse model the efficacy of PSII conjugated to recombinant toxins A (TcdA-B2) and B (TcdB-GT) fragments as carriers. Both glycoconjugates induced anti-PSII IgG but TcdB-GT conjugate was the most potent (Romano et al., 2014).

Reid et al. (2012) identified in all *C. difficile* strains tested, a carbohydrate polymer with the structure of a lipoteichoic acid (LTA) also called PS-III. Cox et al. (2013) examined LTA as a vaccine candidate. Two carrier proteins were used either human serum albumine (HSA) or a genetically inactivated *Pseudomonas aeruginosa* exotoxin A (ExoA). The attachment point for conjugation was an amino group present at the *N*-acetyl-glucosamine residues within the LTA polymer-repeating unit. These conjugates were used to immunize by parenteral route rabbits and mice. Immune sera recognized live vegetative cells and spore forms in an immunofluorescence assay, confirming that the LTA polymer is a highly conserved surface polymer of *C. difficile* inducing a specific immune response. The immunogenicity of ExoA-PS-III conjugate was better than that of the HSA-PS-III conjugate. However, the immune sera recognized all strains of *C. difficile* tested and also *C. butyricum*, *C. subterminale,* and *C. bifermentans* but not *C. perfringens*, *C. sporogenes*, *C. barati,* and *C. botulinum* (Cox et al., 2013).

Surface Proteins of the Spore

The first step of pathogenenesis is the ingestion of spores due to contamination. After contamination by spores, germination begins in the intestinal tract. Spores are highly resistant and responsible for persistence and dissemination. The spore envelope comprises the peptidoglycan cortex, a coat composed of structural and enzymatic proteins and the exosporium predominantly composed of proteins such as BclA glycoproteins and Cde cystein-rich proteins (Paredes-Sabja et al., 2014).

PASSIVE IMMUNIZATIONS TARGETING *C. difficile* SURFACE COMPONENTS

The role of the antibody response in CDI outcome has been emphasized particularly for antibodies targeting toxins but also the whole bacterial cells (Kyne et al., 2001). Various passive immunization strategies have been tested to prevent or cure CDI and prevent recurrences. While most of these strategies target the toxins TcdA and TcdB and consequently neutralize the toxin activity, others target the whole bacterial cell or specific surface components (**Table 1**).

Human Polyvalent Immunoglobulins

First, intravenous (i.v.) immunoglobulin therapy has been used in cases of severe and recurrent CDI in infants and adults simultaneously or after antibiotic standard of care treatment.

TABLE 1 | Passive immunization strategies targeting *C. difficile* surface components.

Antigen	Antibody	Route of administration	Schedule	Model	Outcome	Reference
TcdA and TcdB + whole bacterium	Immune whey protein concentrate (WPC-40, Mucomilk)	Oral	Before and after challenge, then every 8 h during 10 days	Hamster	80–90% protection	van Dissel et al., 2005
			Three times daily for 2 weeks after antibiotic treatment	CDI patients	Significant decrease of recurrences	Numan et al., 2007
Formalin inactivated *C. difficile* cells	Immune whey IgG concentrate (CDIW)	Oral	Three times daily, 14 days	Randomized double-blind study in CDI patients	As effective as metronidazole in the prevention of recurrences	Mattila et al., 2008
TcdB-C-ter, inactivated spores, exosporium, inactivated vegetative cells, SLPs	Hyper-immune bovine colostrum TcdB-HBC, Mix1-HBC, Mix2-HBC	Oral	Two days before challenge and throughout experiment	Mouse model of infection and relapse	HBC-TcdB alone or in combination (Mix1 and Mix2-HBC) prevents and treats CDI in mice and reduces recurrences	Hutton et al., 2017
LMW- and HMW-SLPs	Rabbit hyper- immune serum	Oral	Seven hour before challenge, during challenge, then 6, 17, and 24 h after challenge	Hamster	Prolonged survival after challenge but no protection against death	O'Brien et al., 2005
FliC	Mouse hyper- immune serum	Intra-peritoneal	Twenty four hour before challenge	Mouse	Eighty percent of protection	Ghose et al., 2016b

These normal immunoglobulins may contain antibodies against toxins, which after leakage through the inflamed intestinal mucosa may neutralize the toxins. Although intra-venous gamma globulins (IVGG) have been tested in many patients with CDI, the results are varied and make recommendation for use difficult (O'Horo and Safdar, 2009; Diraviyam et al., 2016). Negm et al. (2017) observed differences in TcdA neutralizing efficacy between three commercial immunoglobulin preparations as well as differences in level of specific IgG isotypes against *C. difficile* antigens.

Bovine Antibodies

After the use of polyvalent human immunoglobulins, animals were immunized with *C. difficile* filtrates or whole extracts. Lyerly et al. (1991) orally vaccinated gestating cows with *C. difficile* formalin inactivated culture filtrate and obtained a bovine immunoglobulin G (IgG) concentrate (BIC) to orally passively immunized hamsters. After *C. difficile* challenge, immunized hamsters were protected from disease during BIC administration but died after treatment cessation. These BICs contained neutralizing IgG against toxins and probably to other antigens. These results were the first to demonstrate that passive oral immunization can protect partially against *C. difficile* lethal infection.

The main interest of colostrum antibodies is their stability in the digestive tract (Kelly et al., 1997). Later, van Dissel et al. (2005) immunized cows with killed whole bacterial cells and the two inactivated toxins TcdA and TcdB. They collected the milk of immunized cows to produce an immune whey protein concentrate (Immune WPC-40; Mucomilk). This concentrate contained secretory IgA (sIgA) antibodies recognizing the whole bacterium and the two toxins (van Dissel et al., 2005). *In vitro*, this WPC was able to neutralize the cytotoxicity of toxins. *In vivo*, in the lethal hamster model challenged with the toxigenic *C. difficile* VPI 10463 strain, oral administration by gavage of 1 mL of WPC 3 h before and after *C. difficile* challenge followed by administration every 8 h for 3 days conferred 80–90% protection of hamsters. In contrast to Lyerly et al. (1991), hamsters survived for at least 28 days after the end of treatment. These authors suggest that specific sIgA against the whole bacterial cell may decrease *C. difficile* gut colonization. Then van Dissel et al. (2005) tested WPC-40 as a medical food in humans to prevent relapses. In an uncontrolled cohort study, WPC-40 was tested in 16 patients with CDI, 9 of whom had one or more relapses. After a standard antibiotic treatment, WPC-40 was given three times daily for 2 weeks. For each dose, 5 g of WPC-40 was diluted in flat mineral water and administered orally before each meal. This treatment was well-tolerated without adverse effects. After treatment, no toxin was detected in fecal samples in 14 out of 15 patients and *C. difficile* was no longer detected in stool culture in 9 out of 15 patients. None of the patients experienced another episode of CDI after treatment during the follow-up period (median 333 days). To confirm this first human study, WPC-40 was tested in a larger cohort of 101 patients with CDI (median age 74 years). After at least 10 days of antibiotic treatment, WPC-40 was given orally for 2 weeks. As previously the daily dose was 15 g of WPC. Interestingly, only 10% of patients relapsed within the 60 days follow-up (Numan et al., 2007). The authors concluded

that oral administration of WPC-40 might help in the prevention of relapses. A phase 2 clinical trial has been performed but the results are not yet posted (NCT00177775).

Another team from Finland also produced an immune whey IgG concentrate (CDIW) using the colostrum of cows immunized with formalin inactivated *C. difficile* (Mattila et al., 2008). In a prospective, randomized, double blind study, they compared CDIW (200 ml three times daily) with metronidazole (400 mg three times daily) to prevent relapses in patients with at least two previous episodes of CDI. After 14 days of treatment, metronidazole was effective in 100% of patients (20) compared to 89% (16/18) for CDIW. At the end of the follow-up study (70 days), the number of patients with relapse was not statistically different between the two groups (44% in CDIW treated group, 45% in metronidazole group). The authors concluded that in this preliminary study CDIW was as effective as metronidazole in the prevention of CDI recurrences and was well-tolerated. Unfortunately, the study was early interrupted.

Recently an Australian team developed hyper-immune bovine colostrum (HBC) to prevent and treat CDI (Hutton et al., 2017). These authors used different *C. difficile* components (027 strain DLL3109) to immunize pregnant cows: the TcdB-C-terminal binding domain, inactivated whole spores or an exosporium extract, and inactivated vegetative cells or a SLP-preparation. They obtained different HBC, named TcdB-HBC, Spore-HBC, Exo-HBC, Veg-HBC, and SLP-HBC respectively. In a mouse model of CDI infections (primary infection and relapse), these HBCs were able to prevent and treat CDI. For prophylactic administration HBC was given *ad libidum* 2 days prior infection and throughout the experiment. Mice treated with Veg-HBC or SLP-HBC did not survive infection. In contrast, survival rates were significantly higher in Spore-HBC ($p < 0.0001$), Exo-HBC ($p = 0.0075$), TcdB-HBC ($p < 0.0001$), and vancomycin ($p < 0.0001$) treated mice compared to untreated mice, which died rapidly after *C. difficile* challenge. The survival rate was the highest in vancomycin and TcdB-HBC (70%) treated mice with no significant difference. Then, various mixtures of HBC in equal ratios were tested: Mix1-HBC contained Spore-HBC, Veg-HBC and TcdB-HBC, Mix2-HBC contained Exo-HBC, SLP-HBC, and TcdB-HBC and Mix3-HBC that contained TcdB-HBC diluted 1:3 in non-immune colostrum to correspond to the amount of TcdB-HBC present in Mix1 and 2. Interestingly, Mix1-HBC and Mix2-HBC treated mice had 70 and 80% survival rate respectively, whereas Mix3-treated mice died after 2 days as did control mice without treatment. Survival rates were not significantly different between vancomycin, TcdB-HBC, Mix1-HBC, and Mix2HBC treated mice. Spore enumeration was similar in all groups (10^6 CFU/gram), indicating that these treatments had no effect on colonization. Mix2-HBC was also tested in a mouse disease relapse model. Control mice receiving no colostrum after vancomycin treatment had a low survival rate (11%). In contrast, colostrum-treated mice had a higher survival rate (77%) but always shedded spores in the feces suggesting no effect of this mixture on colonization. These authors concluded "that administration of HBC-TcdB alone or in combination with spore or vegetative cell-targeted colostrum prevents and treats CDI in mice and reduces recurrence."

One of the main advantages of HBC is the oral administration, which offers several advantages as compared to parenteral administration such as easy administration, local activity in the intestinal tract, and lower cost of production.

Taken together these results suggest that it could be interesting to raise antibodies specific to surface components of the vegetative cell or the spore to unravel the role of specific-surface component antibodies in preventing intestinal colonization.

Specific Antibodies to *C. difficile* Surface Components

Anti SLP Antibodies

Several studies suggest that the capacity to induce an immune response against SLPs may influence CDI outcome. O'Brien et al. (2005) produced rabbit polyclonal antibodies against SLPs. The SLPs used for immunization were purified from a crude SLP-extract from UK reference strain R13537 (ribotype 1). These antibodies reacted strongly with both LMW- and HMW-SLPs. Then, they tested these antibodies to prevent CDI in the hamster lethal model. Rabbit antiserum was administered in carbonate buffer pH 9.6 to neutralize gastric acid. 100 μl doses were given oro-gastrically to hamster 7 h before challenge, during challenge as a mixture pre-incubated with the *C. difficile* challenge inoculum, then 6, 17, and 24 h of infection. "This passive immunization strategy was unable to delay the onset of clinical symptoms and prevent death" but prolonged survival in *C. difficile* infected hamsters compared to untreated infected hamsters (medium post-challenge survival 156 h compared to controls 75 and 69 h). In addition, the authors demonstrated by an *in vitro* assay that anti-SLP antibodies enhanced *C. difficile* phagocytosis by monocytes. This partial protection could be explained by an inadequate level of antibodies (mainly IgG in this antiserum) reaching the intestinal mucosa.

Single-domain antibodies (VHHs or nanobodies) obtained from the variable domains of *Camelidae* heavy-chain IgGs have the affinity and specificity of corresponding monoclonal antibodies. In addition, they are resistant to extreme pH and proteases and are interesting candidates for oral administration (Péchiné et al., 2017). Kandalaft et al. (2015) produced VHHs targeting the SLPs from *C. difficile* strain 027 QCD-32g58. Specific VHHs were selected from an immune llama VHH phage display library (Kandalaft et al., 2015). VHHs bound QCD-32g58 SLPs with high affinity but also SLPs from other strains of various ribotypes. A few of them were resistant to pepsin at physiological concentration. A combination of three LMW-SLP specific VHHs inhibited *in vitro* motility.

Anti-flagellin Antibodies

Ghose et al. (2016b) immunized mice with *C. difficile* flagellin FliC (strain VPI 10463) and obtained specific hyper-immune serum. In a mouse model of CDI, 400 μl of FliC specific hyper-immune serum was intraperitoneally (i.p.) administered simultaneously with clindamycin administration. Then, 24 h later, mice were challenged with a *C. difficile* strain (UK1). Passive immunization led to protection of 80% of treated mice (four out five) whereas four out five control mice treated with a non-immune serum died between 3 and 7 days post-challenge.

In addition, anti-FliC antibodies were detected in four out of five mice from the challenge until day 10. Since FliC plays a multifactorial role in the pathogenesis, protection elicited by anti-FliC antibody may inhibit various steps of *C. difficile* pathogenesis.

VACCINES TARGETING *C. difficile* SURFACE COMPONENTS

Different vaccine strategies to prevent or cure CDI and recurrences have also been developed and tested in animal models (**Table 2**).

Spore Components as Vaccine Antigens

Clostridium difficile germinates, colonizes and persists in the human gut partly due to spore proteins. Ghose et al. (2016a) used two surface-bound spore proteins, CdeC and CdeM as vaccine candidates against *C. difficile*. The recombinant spore proteins administered i.p. with adjuvant (alum) were immunogenic in mice. Vaccination with high doses of CdeC and CdeM were able to protect mice (100% of protection) against CDI after challenge with *C. difficile* UK1. Vaccine assays in golden Syrian hamsters with the same spore proteins induced a protection of 80% against challenge with *C. difficile* 630Δerm strain (Ghose et al., 2016a). Vaccinated hamsters had serum IgG antibodies against CdeC and CdeM that have to reach the intestinal lumen to neutralize the spore proteins.

Crude Extracts as Vaccine Antigens

Colonization is a crucial step in CDI. Several *C. difficile* surface proteins may contribute to colonization (Deneve et al., 2009) and could be used as vaccine antigens. It was shown many years ago that besides toxins, somatic antigens, and surface components induce an immune response in the host important during the course of infection (Pantosti et al., 1989; Mulligan et al., 1993).

Membrane fraction from non-toxigenic *C. difficile* was used as vaccine candidate. Mice subcutaneously vaccinated with a non-toxigenic *C. difficile* membrane fraction and TiterMax Gold adjuvant, produced serum IgG and intestinal fluid IgA specific to the antigen that were able to inhibit *in vitro* adherence of *C. difficile* to Caco-2 cells (Senoh et al., 2015). In addition, *C. difficile* surface proteins have been evaluated as vaccine antigens in the hamster model to prevent intestinal colonization. A cell wall extract of a non-toxigenic *C. difficile* strain administered intra-rectally with cholera toxin as adjuvant partially prevented death in hamsters after a *C. difficile* challenge (Péchiné et al., 2013).

Cell Wall Proteins as Vaccine Antigens

Ni Eidhin et al. (2008) immunized hamsters i.p. and/or intranasally with LMW and HMW-SLP (crude SLP purified by anion exchange chromatography). Different adjuvants were used: alum, Ribi, cholera toxin, and chitosan glutamate (Ni Eidhin et al., 2008). Immunizations with alum as adjuvant led to an increase of serum anti-SLP IgG. However, anti-SLP IgG titers did not correlate with post-challenge survival. In addition, the

TABLE 2 | Vaccine strategies in animal models targeting *C. difficile* surface components.

Antigen	Adjuvant	Route of administration	Schedule	Model	Outcome	Reference
Spore proteins CdeC, CdeM	Alum	Intraperitoneal	Days 0, 14, 28	Mouse and hamster	Hundred percent protection in mice. Eighty percent of protection in hamsters	Ghose et al., 2016a
Cell wall extract	Cholera toxin	Rectal	Days 0, 15, 30	Hamster	Partial protection against lethal challenge	Péchiné et al., 2013
LMW- and HMW-SLPs	Alum	Intraperitoneal	Days 0, 21, 42	Hamster	No significant protection	Ni Eidhin et al., 2008
SlpA precursor	Cholera toxin	Rectal	Days 0, 15, 30	Mouse and hamster	Significant decrease of intestinal colonization at day 10 after challenge in mice. Partial but not lasting protection in hamsters	Bruxelle et al., 2016
Cwp84	Cholera toxin	Rectal	Days 0, 15, 31	Hamster	Significant increased survival (33%). Absence of colonization in surviving hamsters	Péchiné et al., 2011
Cwp84	Pectin beads	Oral	Days 0, 15, 32	Hamster	Forty percent of protection	Sandolo et al., 2011
FliC	Alum	Intraperitoneal	Days 0, 14, 28	Mouse and hamster	Eighty nine percent of protection in mice 43 to 64% of protection in a dose dependent manner in hamsters	Ghose et al., 2016b
GroEL	Cholera toxin	Nasal	Days 0, 7, 14, 28	Mouse	Significant decrease of intestinal colonization	Péchiné et al., 2013
LTA-CRM197	Alum	Subcutaneous	Days 0, 15, 30	Mouse	Significant decrease of intestinal colonization	Broecker et al., 2016
Pilin	Freund's adjuvant	Subcutaneous	Three immunizations	Mouse	Low antibody response. No protection	Maldarelli et al., 2016

median survival time was not significantly different between the immunized and control groups. Bruxelle et al. (2016) assessed the protective effect of vaccination using the recombinant SlpA precursor from the toxigenic *C. difficile* strain 630 on colonization and survival in mouse and hamster models. Immunization assays were performed by rectal administration of SlpA with cholera toxin as adjuvant. In both models, immunizations induced production of serum specific IgG and IgA. In the mouse model, immunizations elicited production of anti-SlpA sIgA and intestinal *C. difficile* colonization was significantly lower in immunized mice compared to control mice 10 days after challenge. In the hamster model, specific SlpA antibodies conferred a partial but not lasting protection against CDI (Bruxelle et al., 2016). More recently, immunizations have been performed by rectal route in a mouse model of CDI with SlpA associated to FliC as adjuvant. Ten days after *C. difficile* challenge, *C. difficile* intestinal colonization decreased significantly in mice immunized with SlpA and FliC as adjuvant compared to the control group (Bruxelle et al., 2017).

The cysteine protease Cwp84 could have an important role in the physiopathology of *C. difficile* (Péchiné et al., 2005b). In the hamster model of CDI, the protease Cwp84 was assessed as vaccine antigen administered by rectal route with cholera toxin as adjuvant. In the Cwp84 immunized groups, survival was significantly prolonged compared to the control group and enumeration of *C. difficile* in feces showed that surviving animals were not colonized by *C. difficile* (Péchiné et al., 2011). Thereafter, Sandolo et al. (2011) evaluated this Cwp84 protease encapsulated into pectin beads as an oral vaccine candidate in the hamster model. Pectin is non-toxic, resistant to gastric or intestinal enzymes and almost totally degraded by pectinolytic enzymes produced by the colonic microbiota. Three immunizations by the intragastric route were performed with beads encapsulating Cwp84 and compared with a control group receiving unloaded beads. Two days after challenge with spores of a toxigenic strain of *C. difficile*, all the control hamsters died. In contrast, 40% of hamsters vaccinated with Cwp84-loaded beads survived 10 days after challenge, proving that oral vaccination with Cwp84 leads to partial protection (Sandolo et al., 2011).

Flagellar Proteins as Vaccine Antigens

After passive immunization of mice using anti-FliC polyclonal serum confirmed protection to be antibody-mediated, Ghose et al. (2016b) performed vaccine assays by the parenteral route in mice and hamsters. Administered i.p. with alum as adjuvant, FliC afforded partial protection (around 50%) against CDI and death in hamsters challenged with *C. difficile* 630Δerm. Interestingly, these immunizations had no adverse effect on the digestive microbiota. In mice i.p. immunized with recombinant FliC, "the protection from CDI (UK1 *C. difficile* strain) and death correlated with a dose response and the number of immunizations received" (Ghose et al., 2016b). The highest protective efficacy of 89% (eight out nine mice disease free) was obtained with three doses of 25 μg of FliC. Notably, immunized mice shed significantly lower levels of spores as compared to non-immunized mice.

Recently, Potocki et al. (2017) combined approaches "to adsorb and display the *C. difficile* flagellar cap protein FliD on the surface of recombinant IL-2-presenting spores of *Bacillus subtilis*." Intranasal immunizations of mice led to the development of a FliD-specific immune response. However, no protection assays in animal models have been performed with this strategy (Potocki et al., 2017).

GroEl as Vaccine Antigen

Among cell surface proteins of *C. difficile*, the heat-shock protein GroEl has been tested as vaccine candidate for immunization against CDI. Intranasal immunization of mice with the recombinant protein GroEL and cholera toxin as adjuvant led to a significant decrease of *C. difficile* intestinal colonization after challenge compared to the control group (Péchiné et al., 2013).

Polysaccharides and Lipoteichoic Acid as Vaccine Antigens

Different vaccine candidates have been evaluated in the mouse model for their immunogenicity. Firstly, a synthetic PS-I repeating unit conjugated to the diphtheria toxoid variant CRM197 was immunogenic in mice and induced immunoglobulin class switching. Second, the disaccharide Rha-(1→3)-Glc has been identified by microarray screening as a minimal epitope. Therefore, a CRM197-Rha-(1→3)-Glc disaccharide conjugate was evaluated. It was able to elicit antibodies recognizing the *C. difficile* PS-I pentasaccharide. These authors concluded "that the synthetic PS-I pentasaccharide repeating unit as well as the Rha-(1→3)-Glc disaccharide could be promising vaccine candidates against *C. difficile*" (Martin et al., 2013).

A parenteral vaccine composed of PS-II conjugated to keyhole lympet hemocyanin (KLH) led to the protection of 90% of mice after challenge with *C. difficile* spores (Monteiro, 2016).

LTA was identified as a possible vaccine antigen to prevent colonization. Broecker et al. (2016) reported on the potential of synthetic LTA glycans as vaccine candidates. They identified LTA-specific antibodies in the blood of *C. difficile* patients. Then, they evaluated the immunogenicity of a semi-synthetic LTA-CRM197 glycoconjugate. Mice were immunized subcutaneously three times with the conjugate associated to alum as adjuvant. This conjugate elicited LTA-specific antibodies in mice that recognized natural LTA epitopes on the surface of *C. difficile*. After oral challenge with *C. difficile*, the degree of bacterial colonization was significantly reduced as compared to control groups (Broecker et al., 2016).

Pili as Vaccine Antigens

Maldarelli et al. (2014) described nine pilin or pilin-like protein genes. Six proteins were purified and used to immunize mice. "Immunizations of mice led to antibody responses that varied in titer and cross-reactivity, a notable result given the low amino acid sequence identity among the pilins" (Maldarelli et al., 2014). More recently these authors immunized mice with various pilins, whether combined or as individual proteins. Unfortunately, "low anti-pilin antibody titers and no protection upon *C. difficile* challenge were observed" (Maldarelli et al., 2016).

CONCLUSION

The two main strategies in the development of specific immunotherapeutics against *C. difficile* target either the toxins or surface components. While neutralizing toxins can prevent clinical signs, an immunotherapy targeting surface components aims to prevent the first step of *C. difficile* pathogenesis gut colonization. Passive immunization with BIC has shown promising results in preventing recurrences in humans. A main advantage of BIC is the oral route of administration that allows delivery of antibodies directly in the colon to neutralize *C. difficile*. Given the multifactorial aspect of *C. difficile* pathogenesis, another strategy to broaden the protection is to target both toxins and colonization factors. Passive immunotherapy has the advantage of rapidity of action, which is essential for treating severe CDI. In contrast, the half-life of antibodies is short and the length of protection is restricted to a period of time. One advantage of vaccination is to develop long-term protection for patients at risk of CDI. Several assays of vaccine targeting surface components of *C. difficile* vegetative cells and spores have been performed in animal models. Some have given interesting results of protection against primary or recurrent CDI but most of the time protection was only partial. Combining several *C. difficile* surface components as vaccine antigens has the advantage of inhibiting several colonization factors involved in the colonization process and consequently could lead to an improved protection. The immune response against *C. difficile* has to be locally effective in the intestinal mucosa, hence the interest of developing vaccine by the mucosal route. Different antigen delivery systems and adjuvants have been tested in animal models to vaccinate by mucosal route against *C. difficile*. To date, only one strategy tested in phase 1 clinical trial aims to elicit both mucosal and systemic immune response to *C. difficile*. This vaccine is a combination of toxoid antigen and a spore component expressed on the surface of inactivated *Bacillus subtilis* spores (CDVAX) (NCT02991417, no result posted).

To conclude, strong scientific evidence supports the development of immunotherapies against *C. difficile* to prevent primary and recurrent CDI and to reduce gut colonization. However, the development of an effective vaccine against *C. difficile* faces many challenges. A key factor is the choice of the antigen. Targeting a panel of antigens could broaden the neutralization of *C. difficile* virulence factors and lead to potent therapeutic efficacy.

AUTHOR CONTRIBUTIONS

AC and CJ wrote the paragraphs on surface components and passive immunization strategies. SP and JB wrote the paragraph on vaccines. All the authors were involved in concept and design of the manuscript and reviewed and edited the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 25 June 2018
doi: 10.3389/fmicb.2018.01382

Application of Antibody-Mediated Therapy for Treatment and Prevention of *Clostridium difficile* Infection

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Clostridium difficile causes antibiotic- and healthcare-associated diarrhea, which is characterized by a high mortality rate (5–15%) and high recurrence rate of 20% or more. Therapeutic alternatives to antibiotics are urgently needed to improve the overall cure rate. Among these, therapeutic antibodies have shown promising results in clinical studies. Herein, the authors review current monoclonal and polyclonal anti- *C. difficile* antibodies that have entered the clinical development stage, either for systemic administration or by the oral route. The antibodies can be applied as monotherapy or in combination with standard-of-care to treat an infection with *C. difficile* or to protect from a recurrence. Bezlotoxumab is the first antibody for secondary prevention of recurrence of *C. difficile* infection approved by the regulatory agencies in US and Europe. The human monoclonal antibody is administered systemically to patients receiving oral standard-of–care antibiotics. Other antibodies are currently in the clinical pipeline, and some are intended for oral use. They show a good safety profile, high efficacy and low production costs, and can be considered promising therapies of the future. The most promising orally administered drug candidate is a bovine antibody from hyperimmune colostral milk, which is in an advanced clinical development stage. Which antibody will enter the market is dependent on its bioavailability at the site of infection as well as its activity against *C. difficile* toxins, protection against colonization and possible action on spore formation. The antibody must demonstrate a clear benefit in comparison with other available treatment options to be considered for use by clinicians.

Keywords: ***Clostridium difficile*, antibody, therapy, systemic, oral**

OPEN ACCESS

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *03 April 2018*
Accepted: *06 June 2018*
Published: *25 June 2018*

Citation:
Förster B, Chung PK, Crobach MJT and Kuijper EJ (2018) Application of Antibody-Mediated Therapy for Treatment and Prevention of Clostridium difficile Infection. Front. Microbiol. 9:1382. doi: 10.3389/fmicb.2018.01382

INTRODUCTION

Clostridium difficile (CD) is an anaerobic spore-forming bacterium that can be present in the gut of asymptomatic CD carriers (Crobach et al., 2018). However, it is also the most important cause of infectious antibiotic- and healthcare-associated diarrhea (Rupnik et al., 2009; Magill et al., 2014). In patients developing symptomatic *C. difficile* infection (CDI), symptoms range from mild diarrhea to fulminant life-threatening colitis (Smits et al., 2016).

The conventional treatment of CDI consists of vancomycin or metronidazole, which are non-selective and lead to further disruption of normal gut microbiota, consequently placing patients at risk for relapse or recurrence. Approximately 25% of CDI patients experience recurrent disease, which is often difficult to treat (Cornely et al., 2012). Alternatively, fidaxomicin is considered to be a specific anti-*C. difficile* antibiotic, but its application is restricted due to high costs. Recently, antibody-mediated therapies for treatment or prevention of CDI have gained attention as alternatives to antibiotic treatment. Here, we discuss the rationale behind antibody-mediated therapy and describe the diverse antibody-mediated therapies (excluding active immunization by vaccines) that are currently studied in the clinic for treatment or prevention (of recurrences) of CDI in humans.

ROLE OF HUMORAL ACTIVITY AGAINST *C. DIFFICILE*

Several studies have demonstrated that the adaptive immune response plays an important role in CDI susceptibility, disease course and risk of recurrence. CDI symptoms are caused by the actions of two large enterotoxins, toxin A (TcdA) and B (TcdB). After the secreted toxins have bound and entered the colonic epithelium, TcdA and TcdB can induce an inflammatory response characterized by chemokine and cytokine production, neutrophil influx, disruption of tight junctions, fluid secretion and cell death (Péchiné and Collignon, 2016; Smits et al., 2016). Some strains, including the more virulent ribotypes 027 and 078, also produce a third toxin, termed binary toxin or CD transferase (CDT). CDT is suggested to cause microtubule-based protrusions on epithelial cells, thereby increasing the adherence of CD to target cells (Smits et al., 2016; Aktories et al., 2018). However, its definite role in pathogenesis remains unclear. CD surface proteins, which mainly act as adhesins, include S-layer proteins, flagellar proteins FliC and FliD and cell wall proteins (Cwps) such as Cwp84. These surface proteins also contribute to the initiation of the inflammatory response via cytokine production and interaction with toll-like receptors (Péchiné and Collignon, 2016). Inflammation induced by the innate immune response promotes an adaptive immune response with memory.

Both antibodies to CD toxins and surface proteins have been described, and the adequacy of the humoral immune response can influence susceptibility to CD colonization and/or progression to CDI. In 55 CDI patients, significantly higher serum IgM anti-SLP (S-layer proteins) antibody levels were found at the initial episode in non-CDI recurring patients compared to patients who went on to develop recurrent CDI (rCDI) (Drudy et al., 2004). Furthermore, serum antibody levels for surface proteins, including FliD and FliC, were significantly higher in control patients than in CDI patients (Péchiné et al., 2005). Therefore, antibodies against CD surface proteins are thought to have a protective role against (re)colonization.

Anti-TcdA and anti-TcdB antibodies can protect colonized patients from progression to CDI or recurrent disease. Anti-toxin antibodies are present in the general population, and antitoxin seropositivity prevalence in the general population of 24 or 66% have been previously reported (Viscidi et al., 1983; Bacon and Fekety, 1994). Anti-TcdA antibody levels have also been studied in hospitalized patients receiving antibiotic treatment. After intestinal colonization with *C. difficile*, greater increases in serum levels of anti-TcdA IgG were reported in those patients who remained asymptomatic, compared to those who progressed to CDI. Serum anti-TcdA IgG increased rapidly after colonization, indicating the presence of a systemic anamnestic response to TcdA after day three (Kyne et al., 2000). Furthermore, the development of higher serum anti-TcdB and anti-TcdA IgG levels and fecal anti-TcdA IgA levels during the course of CDI were all reported to be associated with a lower risk of rCDI (Aronsson et al., 1985; Warny et al., 1994). In another study, higher anti-TcdA IgM and IgG levels at day 12 were associated with a lower risk of recurrence (Kyne et al., 2001). Anti-toxin antibodies may be directly protective against disease by neutralizing secreted toxin. The association of anti-toxin antibodies with decreased recurrence risk may be explained by their ability to remove the toxins and stabilize the gut epithelium and microbiota (Dieterle and Young, 2017).

ANTIBODIES FOR SYSTEMIC ADMINISTRATION TO PREVENT (RELAPSING) CDI

Actoxumab and Bezlotoxumab

In 2017, bezlotoxumab (Zinplava©) was approved by the Food and Drug Administration (FDA), and 1 year later by the European Medicines Agency (EMA), as the first monoclonal antibody for prevention of rCDI in patients ≥18 years old.

Actoxumab and bezlotoxumab were developed in parallel as fully human monoclonal IgG (HuMAbs) directed against TcdA and TcdB respectively. Inactivated TcdA and TcdB (both from Techlab), and additionally recombinant TcdB, were used as antigens to immunize mice transgenic for human immunoglobulin genes (HuMAb mice, Medarex Inc., Bloomsbury, New Jersey) (Babcock et al., 2006). Various hybridomas were obtained and screened *in vitro* and *in vivo*. The lead candidate against TcdA (CDA1) recognized the C-terminal region of TcdA and could prevent binding to a cognate receptor on the surface of target cells. The lead candidate against TcdB (MDX-1388) was proposed to bind to the N-terminal domain (putative cell-binding region) of TcdB (**Table 1**).

A combination treatment with CDA1 and MDX-1388 in a hamster model for primary disease and relapse showed the best outcome. After safety and pharmacokinetics of CDA1 were assessed in healthy volunteers in a phase I clinical trial (Taylor et al., 2008), efficacy of combination treatment with CDA1 and MDX-1388 was examined in a randomized double-blind, placebo-controlled phase II clinical trial (Lowy et al., 2010). The addition of both antibodies to either metronidazole- or vancomycin-treated patients with CDI significantly reduced recurrence rates. The recurrence rate of patients in the treatment group was 7%, compared to 25% in the placebo group (**Table 2**). No significant differences were found in disease severity, number

TABLE 1 | Clinically tested antibody-mediated therapeutic agents for CDI.

Agent	Manufacturer	Type of antibody	Mode of action	State of development	Indication
SYSTEMIC APPLICATION ROUTE					
Actoxumab	Merck Sharp & Dohme Ltd (Europe), Inc.	Antitoxin A (CDA1, MK-3415) human monoclonal IgG antibody	Prevents binding to a cognate receptor	Terminated after phase III trial	None
Bezlotoxumab	Merck Sharp & Dohme Ltd (Europe), Inc.	Antitoxin B (CDB1, MDX-1388, MK-6072) human monoclonal IgG antibody	Prevents binding to a cognate receptor	FDA and EMA approved	rCDI
IVIG	Various	Human IgG	Neutralization of TcdA/TcdB	Market (for other indications)	rCDI/CDI/severe CDI
ORAL APPLICATION ROUTE					
IgAbulin	ImmunoAG Vienna/Baxter	Human IgA	Neutralization of TcdA/TcdB	Market (for other indications)	CDI
Oral hyperimmune bovine immunoglobulin concentrate (BIC)	Beth Israel Deaconess Medical Center	Polyclonal antibody-enriched colostral immune whey protein concentrate	Neutralization of TcdA/TcdB	Clinical phase I	CDI
MucoMilk	MucoVax BV	Polyclonal antibody-enriched milk immune whey protein concentrate	Neutralization of TcdA/TcdB, Binding of vegetative bacterial cells	Clinical development	rCDI
Cediff	Novatreat Ltd.	Polyclonal antibody-enriched colostral immune whey protein concentrate	Binding of toxigenic vegetative bacterial cells	Interrupted in clinical phase II	rCDI
IMM-529	Immuron Ltd.	Immune colostrum concentrate	Neutralization of TcdB, Binding of vegetative bacterial cells and endospores	Clinical phase I/II	CDI/rCDI

IVIG, intravenous immunoglobulin; TcdA, C. difficile toxin A; TcdB, C. difficile toxin B; CDI, C. difficile disease; rCDI, recurrent CDI.

of days to resolution, or proportion of treatment failure. Adverse events were reported to be similar in both groups.

The worldwide development and commercialization rights for both antibodies were purchased by Merck for US$ 60 million, (Markham, 2016) and two double-blind, placebo-controlled phase III trials, MODIFY I and II, were conducted to assess efficacy and safety of both antibodies (subsequently named actoxumab and bezlotoxumab) (Wilcox et al., 2017). MODIFY I had an adaptive design, allowing enrolment cessation in any group after interim analysis. Patients were randomly assigned in a 1:1:1:1 ratio to either receive a single dose of bezlotoxumab, actoxumab, bezlotoxumab plus actoxumab or placebo in addition to standard antibiotic treatment for CDI. During the interim analysis of MODIFY I, higher mortality and more serious adverse events were found in the actoxumab group, causing enrolment in this group to be terminated. Subsequently, actoxumab was not evaluated in MODIFY II. While in both trials initial cure rates did not differ between antibody-treated groups and placebo groups, significantly lower recurrence of CDI was found in the bezlotoxumab groups during 12 weeks post-treatment, as recurrent infections were seen in 17 and 16% in these groups in MODIFY I and II respectively, in comparison to 28 and 26% in the placebo groups (**Table 2**). Subgroup analysis of initially cured participants similarly showed significant differences in the recurrence rate, while sensitivity analysis taking into account missing data and early discontinuations were consistent with these findings. The addition of actoxumab to bezlotoxumab did not improve efficacy in MODIFY I. The overall safety profile of bezlotoxumab was favorable, though the assessment reports of FDA and EMA noted a numerical increment of heart failure and all-cause mortality among bezlotoxumab treated subjects with baseline congestive heart failure, compared to placebo-treated patients.

The benefit of bezlotoxumab and actoxumab-bezlotoxumab was confirmed in subpopulations at high risk for rCDI or CDI complications. *Post-hoc* analyses of the two phase 3 trials (MODIFY 1 and MODIFY II) showed greater benefit of bezlotoxumab than placebo in patients with at least three risk factors. No difference in CDI recurrence was observed for patients with no predefined risk factors (Gerding et al., 2018).

Bezlotoxumab is available for the retail price of $4,560 per vial (Lee et al., 2017). In a model-based analysis of the MODIFY trial subjects by the manufacturer, bezlotoxumab was reported to be cost-effective compared to placebo (Prabhu et al., 2018), although no reports of comparison to other approaches such as fecal transplant or fidaxomicin treatment have been published.

Intravenous Immunoglobulin

In contrast to monoclonal antibody therapies, a cocktail of synergistic polyclonal antibodies has the ability to bind multiple

TABLE 2 | Studies evaluating antibody-mediated therapeutics in humans.

Agent	Study (author, year)	Study design	Efficacy
SYSTEMIC APPLICATION ROUTE			
Actoxumab (CDA1)	Wilcox et al., 2017	*Clinical Phase III study* MODIFY I:	
		Addition of actoxumab, bezlotoxumab, a-b or placebo to SoC treatment in adult (r)CDI patients	Higher mortality and more AE in actoxumab group; evaluation terminated
Bezlotoxumab (MDX1388)	Wilcox et al., 2017	*Clinical Phase III study* Addition of bezlotoxumab ($n = 781$) vs. placebo ($n = 773$) to SoC treatment in adult (r)CDI patients	
		MODIFY I	No difference initial cure rate (77% vs. 83%) Significant lower rCDI (17% vs. 28%)
		MODIFY II	No difference initial cure rate (83% vs. 78%) Significant lower rCDI (16% vs. 26%)
Actoxumab-bezlotoxumab	Lowy et al., 2010	*Clinical Phase II study* Addition of CDA1+ and MDX1388 in single infusion ($n = 101$) vs. placebo ($n = 99$) to SoC treatment for CDI	Significant lower laboratory-documented rCDI: 7% comparator vs. 25% placebo
	Wilcox et al., 2017	*Clinical phase III study* Addition of actoxumab-bezlotoxumab ($n = 773$) vs. placebo ($n = 773$) to SoC treatment in adult (r)CDI patients	
		MODIFY I	No difference initial cure rate (75% vs. 83%) Significant lower rCDI(16% vs. 28%)
		MODIFY II	No difference initial cure rate (72% vs. 78%) Significant lower rCDI (15% vs. 26%)
IVIG	Leung et al., 1991	*Case series* 5 pediatric patients with hypoglobulinaemia and rCDI, 400 mg/kg IVIG	All patients had full resolution of symptoms
	Wilcox, 2004; McPherson et al., 2006; Negm et al., 2017	*Retrospective studies* 36 adult patients with (r)CDI, 150–500 mg/kg IVIG	24 patients showed therapeutic response, 12 did not respond
	Juang et al., 2007; Shahani and Koirala, 2015	*Retrospective studies* 39 adult patients with severe CDI, 82 control patients, 400 mg/kg IVIG	No significant differences regarding outcome and severity of symptoms
ORAL APPLICATION ROUTE			
IgAbulin	Tjellström et al., 1993	*Case study* 1 pediatric patient with severe CDI	Full resolution of symptoms
MucoMilk	van Dissel et al., 2005	*Prospective cohort study* 15 adult patients, 1 pediatric patient with completed antibiotic therapy, under which 9 patients with rCDI	No relapse within a median follow-up period of 333 days
	Numan et al., 2007	*Prospective cohort study* 101 adult patients, with completed antibiotic therapy, 61 patients with CDI 40 patients with rCDI (40%)	Prevention of relapse by 50% during a follow up period of 60 days
Cediff	Mattila et al., 2008	*Prospective study* 38 adult patients with rCDI 20 were treated with Cediff, 18 were treated with metronidazole	Cediff was as effective as metronidazole in the prevention of CDI recurrences during a 70 day follow up (sustained recovery 56% vs. 55%)

a-b, actoxumab-bezlotoxumab; AE, adverse event; SOC, standard-of-care; CDI, C. difficile disease; CDA1, human monoclonal anti-toxin A; CDB1, human monoclonal anti-toxin B; rCDI, recurrent CDI; SoC, standard-of-care antibiotic treatment for CDI; TcdA, C. difficile toxin A; TcdB, C. difficile toxin B; vs., versus; IVIG, intravenous immunoglobulin.

targets and mediate a variety of effector functions (Wang et al., 2013).

Intravenous immunoglobulin (IVIG) is in use for numerous clinically approved pathological conditions (Hässig, 1967; Stiehm et al., 2008), and in more than 100 different indications as an off-label drug (João et al., 2018). The rationale to apply IVIG to patients with CDI is based on the prevalence of serum antibodies against TcdA and TcdB in healthy blood donors (Viscidi et al., 1983; Bacon and Fekety, 1994), and a low antitoxin serum level in patients that are predisposed for recurrent, prolonged or severe CDI (Salcedo et al., 1997; Kyne et al., 2000; **Table 1**). After a successful study in children (Leung et al., 1991) IVIG has been used in several small, mostly retrospective and non-randomized studies with elderly patients suffering from recurrent (Beales, 2002; Wilcox, 2004) or severe CDI (Salcedo et al., 1997; McPherson et al., 2006; Juang et al., 2007; Shah et al., 2014;

Shahani and Koirala, 2015; **Table 2**). So far, there is a lack of robust evidence of a beneficial therapeutic effect of IVIG due to the absence of a sufficiently powered randomized controlled trial (Negm et al., 2017). In 2005, a randomized placebo- controlled interventional study planned for 40 patients with severe relapsing or refractory CDI (ClinicalTrials.gov NCT00177970) was started and prematurely terminated. The sponsor states that they were unable to receive IVIG free of cost from a pharmaceutical company to continue.

In addition to the lack of consensus regarding optimal dose and timing of IVIG administration and patient selection, the quality of IVIG is a pivotal factor. Plasma or blood of 1,000–100,000 healthy donors is used for IVIG manufacturing and the compositions of plasma donor pools are not standardized. Recently, three commercially available IVIG preparations were screened for their reactivity to a panel of seven CD antigens. They showed significant differences in antigen binding and toxin neutralization, which are considered as the predominant mode of action of IVIG against CDI (Negm et al., 2017). Selected sourcing of donors with naturally occurring anti-toxin antibodies for the manufacturing of IVIG with standardized elevated specific antibody levels is feasible (Wasserman et al., 2016). It is questionable if the market size of severe and rCDI and predominance of low-cost medication can offer an incentive to invest into a specialized IVIG.

As a blood-derived product, IgG pool carries a potential (low) risk for blood-borne disease transmission (Chapel, 1999) and contains batch-to-batch variability. Additionally, only a small fraction of antibodies bind the target of interest. The biodistribution of IVIG outside the bloodstream is poorly understood (Brandtzaeg and Baklien, 1976; Wasserman et al., 2012; Nikpoor et al., 2015), and it is unclear if the concentration of antibody that reaches the site of infection could be sufficient to exert the desired effect.

Furthermore, IVIG may not be cost-effective and would not relieve the financial burden on health care. While each CDI episode costs $3,500–5,042 (Dubberke et al., 2008), each IVIG administration is calculated at $4551.25 (Blackhouse et al., 2010).

ANTIBODIES FOR ORAL APPLICATION AGAINST CDI

Oral delivery of antibodies to target pathogens restricted to the gastrointestinal tract provides a highly attractive treatment strategy. A needle-free application directly on the affected site may result in a higher efficacy with reduced side-effects at a lower dose (Jones and Martino, 2016). Human and bovine antibodies have been used in the past to treat CDI.

Human Antibodies

A human IgA and IgG pool (Tjellström et al., 1993; Saturno, 2006) derived from healthy blood donors was successfully used to orally treat children with severe CDI resistant to standard treatment (**Table 2**). Consistent with findings for infants orally treated with human IgG (Blum et al., 1981), IgA remained restricted to the gastrointestinal tract and did not enter the blood stream (Casswall et al., 1996).

Bovine Antibodies

Hyperimmune bovine colostrum (HBC) or milk is an easily scalable and cost-effective source (USD$1/gram antibody; Hutton et al., 2017) for orally applicable antibodies with a strong safety profile. It is produced by vaccination of cows during gestation, and is rich in targeted IgG or secretory IgA (sIgA).

HBC derived from cows that were immunized with recombinant mutants of TcdA and TcdB showed a therapeutic efficacy in gnotobiotic piglets with diarrhea due to CDI (Sponseller et al., 2015).

A single-site, open, crossover, clinical phase I study on six healthy volunteers was performed to analyse the survival of bovine IgG in the human gastrointestinal tract after application of a single oral dose. Antibody-enriched colostral immune whey protein concentrate (BIC) was derived from cows immunized with either a toxoid derived from culture medium of toxigenic CD containing both TcdA and B, or a toxoid of purified TcdA (Kelly et al., 1997). Antibody within enteric coated capsules could be recovered to about 30% of the initial dose in stool while retaining its specific toxin-neutralizing activity. Based on *in vitro* and preclinical findings, it was assumed that the bovine IgG could neutralize CD toxins within the colonic lumen (Kelly et al., 1997). This study also claimed bovine IgG concentrate against CD to be safe for oral use in humans. The following single site, open, phase I study was performed on six volunteers with an end ileostomy to examine safety and bioavailability of a single oral dose. This study showed the recovery of about 50% of the orally applied antibody and a retained activity. It also revealed the difficulty of properly formulating the oral antibody therapy for patients with abnormal mouth to ileum transit times (Warny et al., 1999).

Immune whey protein concentrate derived from mature milk of cows vaccinated with formaldehyde-inactivated whole CD cells (VPI10463) and toxoid prepared from the CD culture filtrate contained a high concentration of specific sIgA antibodies (Schmautz et al., 2018; **Table 1**). This product, named MucoMilk, was intended to serve as "clinical nutrition" (not medication) for patients with CDI and considered to be safe (Young et al., 2007). The prevention of relapses of CDI was evaluated in a prospective uncontrolled cohort study enrolling 16 patients with CDI. The whey concentrate was applied subsequently to standard antibiotic treatment. During a follow-up period of median 333 days, none of the patients had experienced a relapse (van Dissel et al., 2005). A following non-blinded, clinical cohort study applying the same treatment scheme on 101 patients showed a reduction of recurrence by about 50% (van Dissel et al., 2005; Numan et al., 2007; Bauer et al., 2008; Bauer and van Dissel, 2009; **Table 2**).

Next, a controlled, double-blind, randomized, parallel-group, multicenter trial with concentrated colostral immune whey from cows vaccinated with formaldehyde-inactivated whole CD cells (T-37067, T-36842), named Cediff, was performed (**Table 1**). Cediff was compared with metronidazole against rCDI in patients that already encountered at least one episode. Although only a small number of patients were included, the recurrence rate of both treatment groups was similar (Mattila et al., 2008; **Table 2**). Further development was stopped.

Recently, a phase 1/2, randomized, double blind, placebo-controlled clinical study started enrolling patients with primary and rCDI, to evaluate safety and tolerability of hyper-immune

bovine colostrum powder (IMM-529) together with standard-of-care (ClinicalTrials.gov NCT03065374). IMM-529 is derived from cows vaccinated with TcdB, vegetative cells and endospores of CD (Hutton et al., 2017; **Table 1**). In contrast to previous approaches (Korhonen et al., 2000) the immune colostrum powder is produced as pharmaceutical.

PERSPECTIVE

Further antibodies against CDI are in preclinical development, such as avian antibodies against TcdA and B (Kink and Williams, 1998) or spores (Pizarro-Guajardo et al., 2017) and fully human polyclonal IgG obtained by immunization of transgenic cattle with a trivalent and quadravalent toxin vaccine (Tian et al., 2017).

Smaller antibody formats such as a tetravalent bispecific heavy-chain-only single domain (VHH) antibody against TcdA and B features neutralization in a subnanomolar range and efficacy in animal models of CDI and is deliverable by gene therapy (Yang et al., 2014, 2016). Engineered lactobacilli that produce variable domains of heavy chain only antibodies against CD toxins show partial protection in a hamster model (Andersen et al., 2016).

Fully functional antibodies that consist of heavy chains only are named nanobodies. Nanobodies against binary toxin (Unger et al., 2015) and surface proteins of CD (Kandalaft et al., 2015) as well as variable domains derived from cartilaginous shark IgNAR (Krah et al., 2016) have shown effects *in vitro*. A camelid single domain antibody against TcdA could be easily optimized by an affinity maturation platform and could serve as an alternative therapeutic modality in the future (Sulea et al., 2018). A novel humanized monoclonal antibody recognizes a single, highly conserved epitope on the TcdB glucosyltransferase domain and neutralizes TcdB from various CD strains (Kroh et al., 2018).

CONCLUSION

Taken together, antibodies such as bezlotoxumab have proven to be capable of protecting from rCDI, while mostly being directed against toxins. A better understanding of virulence factors of CD could help to broaden the repertoire of therapeutic targets and may result in antibodies also applicable for severe and refractory CDI or CDI caused by "hypervirulent" strains. Due to their property of maintaining the integrity of the gut microflora and providing no selective pressure for escape mutants (Steele et al., 2013), polyclonal antibodies could act synergistically with antibiotics and FMT and could easily be integrated in treatment regimens, provided that their costs are competitive with current therapies. Oral antibodies have the clear advantage that they could be applied not only at the clinic but also at the patient's home.

AUTHOR CONTRIBUTIONS

BF, PC, MC, and EK have written the manuscript and compiled the tables.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 12 June 2018
doi: 10.3389/fmicb.2018.01242

Mechanistic Insights in the Success of Fecal Microbiota Transplants for the Treatment of *Clostridium difficile* Infections

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Fecal microbiota transplantation has proven to be an effective treatment for infections with the gram-positive enteropathogen *Clostridium difficile*. Despite its effectiveness, the exact mechanisms that underlie its success are largely unclear. In this review, we highlight the pleiotropic effectors that are transferred during fecal microbiota transfer and relate this to the *C. difficile* lifecycle. In doing so, we show that it is likely that multiple factors contribute to the elimination of symptoms of *C. difficile* infections after fecal microbiota transplantation.

Keywords: fecal microbiota transplant, *Clostridium difficile* infection, short chain fatty acids, bile acids, sialic acids, antimicrobial peptides, bacteriocins, bacteriophages

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *21 April 2018*
Accepted: *23 May 2018*
Published: *12 June 2018*

Citation:
Baktash A, Terveer EM, Zwittink RD, Hornung BVH, Corver J, Kuijper EJ and Smits WK (2018) Mechanistic Insights in the Success of Fecal Microbiota Transplants for the Treatment of Clostridium difficile Infections. Front. Microbiol. 9:1242. doi: 10.3389/fmicb.2018.01242

INTRODUCTION

Fecal microbiota transplantation (FMT), the transfer of (processed) fecal material from healthy donors to patients, has been documented already over 1700 years ago for the treatment of gastrointestinal illness in humans. The Chinese scholar Ge Hong used fecal suspension administered orally to treat severe diarrhea; the 16th century scientist Li Shizhen referred to such suspensions as "yellow soup" (Zhang et al., 2012). Similarly, during World War II, soldiers stationed in North-Africa took up the practice of treating dysentery using camel dung, after observing locals do so (Lewin, 1999). Though in either case the rationale for treatment was unknown, anecdotal reports of successful treatment triggered a renewed scientific interest in the middle of the 20th century.

The first description of a fecal enema as treatment in modern medical literature dates from 1958, when four patients with pseudomembranous colitis were treated (Eiseman et al., 1958). At that time not identified yet as such, the major causative agent of pseudomembranous colitis is *Clostridium difficile*, a gram-positive, spore forming, obligate anaerobic bacterium (Hall and O'Toole, 1935; George et al., 1978; Larson et al., 1978; Smits et al., 2016). It was originally identified as part of the normal gut microbiota of healthy infants in 1935 (Hall and O'Toole, 1935) and was recently reclassified as *Clostridioides difficile*, based on phenotypic, chemotaxonomic and phylogenetic analyses (Lawson et al., 2016). 16S rRNA gene sequence analysis showed that the closest relative is *Clostridium mangenotii* (Lawson et al., 2016). Ground-breaking work in the 70's established that *C. difficile* was a transmissible pathogen that produced toxin(s) capable of inducing gastrointestinal

disease in various animals (Chang et al., 1978; Bartlett, 2009). *C. difficile* infection (CDI) is believed to be triggered by antibiotics which disrupt the normal microbiota enabling the outgrowth of toxin-producing *C. difficile* (Dethlefsen and Relman, 2011; Rea et al., 2011; Hensgens et al., 2012; Keller and Kuijper, 2015).

The first-line therapy of CDI according the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline is with the prodrug metronidazole, that is converted by anaerobic bacteria into nitroso radicals that exert an antimicrobial effect (Debast et al., 2014). However, metronidazole appears less effective compared with vancomycin and fidaxomicin in inducing initial cure (Johnson et al., 2014; Ooijevaar et al., 2018). As a result, vancomycin and fidaxomicin are recommended in the 2017 update of the guidelines of the Infectious Diseases Society of America and Society for Healthcare Epidemiology of America, even for mild and first occurrence CDI (McDonald et al., 2018). Nevertheless, the risk of recurrence after treatment within 8 weeks is 15–25% and rises to 40–65% in patients suffering from multiple recurrences (Fekety et al., 1997; Cornely et al., 2012; Johnson et al., 2014; Keller and Kuijper, 2015).

16S rRNA gene sequence analysis of gut microbiota of patients with an initial or recurrent CDI showed that patients with recurrent disease showed a highly variable bacterial composition in comparison with the normal predominance of Bacteroidetes and Firmicutes. Furthermore, patients with recurrent CDI showed lower species richness compared with patients with an initial episode of CDI patients and control subjects (Chang et al., 2008; Seekatz et al., 2016). This suggested that modulating microbiota composition could be key in the treatment of recurrent CDI.

The first randomized control trial using FMT to treat recurrent CDI (16 patients in the FMT arm versus 26 in the control arms) demonstrated a remarkable efficacy (van Nood et al., 2013), which was confirmed in multiple independent analyses. For instance, the meta-analysis by Quraishi and co-workers, that included seven randomized controlled trials (RCTs) and 30 case series, showed that FMT is more effective than vancomycin in resolving recurrent and refractory CDI with a relative risk of 0.23 and a clinical resolution of 92% (Quraishi et al., 2017). The meta-analysis by Moayyedi and co-workers included ten RCTs with a total of 657 patients with *C. difficile*-associated diarrhea and demonstrated that FMT was significantly more effective compared with placebo or vancomycin treatment, with a relative risk of 0.41 (Moayyedi et al., 2017). However, great heterogeneity exists among the included studies with respect to donor feces volume, FMT preparations, route of administration, pre-treatment and numbers of FMTs (Moayyedi et al., 2017; Quraishi et al., 2017; Terveer et al., 2018). After FMT, patients show an increase in microbiota diversity, reaching levels that are observed in healthy donors (van Nood et al., 2013; Fuentes et al., 2017). Taken together, the data show that for patients suffering from multiple recurrent CDI, FMT is a highly effective treatment (Ooijevaar et al., 2018) and FMT is now indicated in therapeutic guidelines for this group of patients (McDonald et al., 2018). Recommended treatment modalities and FMT procedures have been reviewed elsewhere (Cammarota et al., 2017; Terveer et al., 2017b; Woodworth et al., 2017; Panchal et al., 2018).

Multiple recurrent *C. difficile* infections remain the prime – and arguably only - example for which there is a consistent body of evidence for treatment by FMT and for which FMT is indicated as treatment strategy (Smits et al., 2016; McDonald et al., 2018; Ooijevaar et al., 2018). Yet, what is it in the donor material that results in the elimination of symptoms, and/or detectable presence of the pathogen? Fecal suspensions commonly used for FMT contain a plethora of abiotic and biotic factors. In this Review, we summarize our understanding of the possible active constituents in donor fecal material in relation to the *C. difficile* lifecycle (**Figure 1A**).

COLONIZATION RESISTANCE

The first step in the establishment of an infection is colonization of a host with pathogenic *C. difficile* bacteria. Such colonization can result in asymptomatic carriership, or in disease (Eyre et al., 2013; Terveer et al., 2017a; Crobach et al., 2018). The onset of disease can be triggered through a disruption of the healthy microbiota due to exogenous factors such as antimicrobials (Britton and Young, 2014; Keller and Kuijper, 2015; Smits et al., 2016). In the early 1960's, such antibiotic associated susceptibility to infection was also observed for *Salmonella enterica* (Bohnhoff and Miller, 1962). The hypothesis that a stable microbial community can prevent colonization and/or outgrowth of pathogens has become known as colonization resistance (Lawley and Walker, 2013). In its simplest view, FMT replenishes the microbial diversity that is lost after antimicrobial treatment and therefore confers colonization resistance against *C. difficile*. Though intuitively it is appealing that a dense microbial community exerts this effect, it should be noted that colonization resistance is as poorly defined as FMT. Microbial and immunological factors that play a role in colonization resistance (Lawley and Walker, 2013) are also likely to play a role in the success of FMT. Microbiota-immune interactions have been reviewed elsewhere (Belkaid and Hand, 2014; Khoruts and Sadowsky, 2016), and are not in the scope of this review. Here, we focus on the transferable components of a fecal suspension.

PRIMARY AND SECONDARY BILE ACIDS

If we look beyond numbers, are there any specific mechanisms that might contribute to the efficacy of FMT? What small molecules have been shown to affect the lifecycle of *C. difficile*?

Spores are essential for the transmission of *C. difficile* between hosts and persistence in the (hospital) environment (Deakin et al., 2012; Paredes-Sabja et al., 2014). Upon passing the stomach, bile acids induce germination of *C. difficile* spores (Francis et al., 2013; Kochan et al., 2017) (**Figure 1B**).

Bile acids promote intestinal absorption and transport of lipids, nutrients and vitamins, but also serve a broad range of regulatory functions throughout the body (Chiang, 2009). The

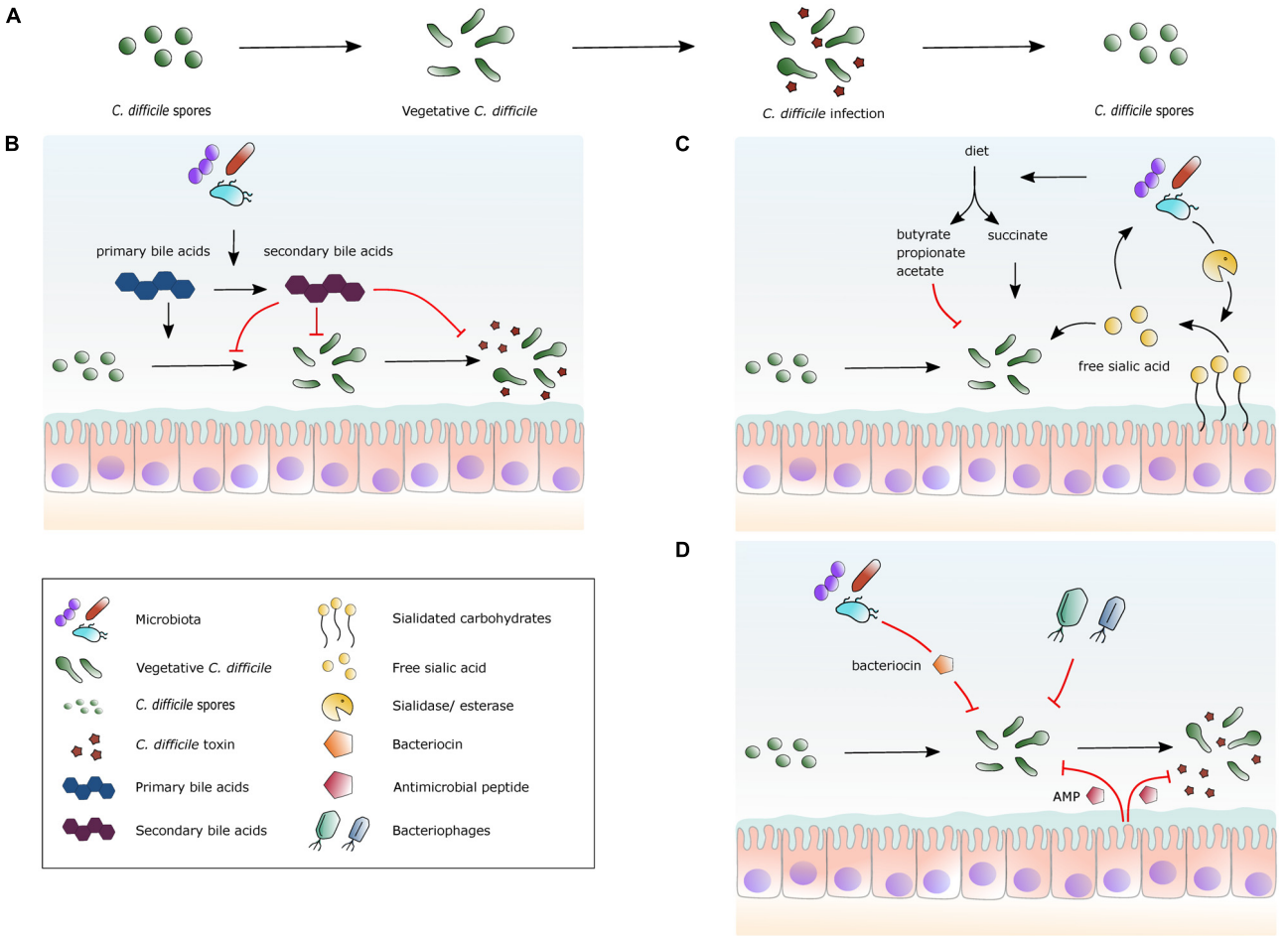

FIGURE 1 | Selected mechanisms by which the gut microbiota, metabolites and peptides can affect *C. difficile*. **(A)** The lifecycle of *C. difficile*. Spores germinate into vegetative cells, which then proliferate and start to produce toxins. The lifecycle is completed by the formation of spores from vegetative cells again. **(B)** A simplified overview of the effects of bile acid metabolism on *C. difficile*. Secondary bile acids are formed from primary bile acids via 7α-dehydroxylation by gut bacteria. Primary bile acids promote germination of spores into vegetative cells, while secondary bile acids generally inhibit this process. Secondary bile acids can inhibit toxin production. **(C)** A simplified view of the effects of specific nutrients on *C. difficile*. Dietary fibers are fermented by the gut microbiota and short-chain fatty acids (SCFAs) are released. Succinate can stimulate *C. difficile* expansion, whereas other SCFAs potentially inhibit growth. Certain bacterial species in the gut cleave sialidated carbohydrates, releasing sialic acid that stimulates *C. difficile* expansion. **(D)** Schematic view of naturally occurring antimicrobial factors. The human gut has a high abundance of microbial species, some of which produce bacteriocins that are bacteriostatic or bactericidal for *C. difficile*. Certain bacteriophages present in the gut microbiota use *C. difficile* as a host. Antimicrobial peptides (AMP) are produced by host cells that inhibit *C. difficile* growth and inhibit toxin activity.

primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized in the liver from cholesterol in a multi-step process and are conjugated to either glycine or taurine (Chiang, 2009). They are hydrophobic and conjugation increases their solubility (Staley et al., 2017). After food intake, bile acids are released into the duodenum and are reabsorbed by active transport in the distal ileum. This enterohepatic circulation has an overall recovery efficiency of ∼95% (Chiang, 2009). The secondary bile acids, deoxycholic acid and lithocholic acid, are formed via 7α-dehydroxylation by gut bacteria from bile acids that are not reabsorbed (Chiang, 2009). These bacteria are members of the Lachnospiraceae and Ruminococcaceae families and include *Clostridium scindens* (Weingarden et al., 2014; Buffie et al., 2015). Recirculated secondary bile acids are conjugated to glycine and taurine similar to primary bile acids (Chiang, 2009).

Bile acids can directly or indirectly influence the composition of the gut microbiota. Several studies demonstrated that bile acids have direct antimicrobial effects by damaging cell membranes and DNA through oxidative and pH stress (Kakiyama et al., 2013; Staley et al., 2017). There is a varying degree of bile resistance, tolerance and susceptibility even within single bacterial species (Staley et al., 2017). Bile acids can also regulate the gut community structure indirectly, as shown for cholic acid (Islam et al., 2011). Supplementation of the diet with cholic acid resulted in an increase of Firmicutes, a group that encompasses bacteria capable of 7α-dehydroxylating cholic acid, and decrease of Bacteroidetes (Islam et al., 2011; Ridlon et al., 2013).

Bile acids in the gastrointestinal tract affect the growth of *C. difficile* (**Figure 1B**). *In vitro*, primary bile acids generally

stimulate germination, whereas secondary bile acids inhibit this process (Francis et al., 2013; Paredes-Sabja et al., 2014; Thanissery et al., 2017). The latter finding is recapitulated in a murine-derived model where physiologically relevant concentrations of primary and secondary bile acids where tested (Theriot and Young, 2015). In this model, *C. difficile* was able to germinate and grow in ileal an cecal content when secondary bile acids were depleted, demonstrating that secondary bile acids inhibit spore germination and growth (Theriot and Young, 2015).

Patients with CDI have an altered fecal bile acid composition in the colon. For instance, secondary bile acids were higher in fecal samples from controls compared to patients with CDI, and primary bile acids were elevated in patients with recurrent disease compared to those experiencing a first episode of CDI (Allegretti et al., 2016).

Fecal microbiota transplantation treatment has a significant effect on the bile acid composition. Pre-FMT fecal samples of CDI patients show a shift in the balance between primary bile acids and secondary bile acids, almost completely toward the former, whereas post-FMT fecal samples contain predominantly secondary bile acids, similar to donor samples (Weingarden et al., 2014). A combination of pre-FMT bile acids induced germination of *C. difficile in vitro*, whereas the post-FMT combination did not. Likewise, pre-FMT bile acids did not affect the vegetative growth of *C. difficile*, but bile acids from post-FMT feces significantly reduced growth (Weingarden et al., 2016b). Differences in bile acid composition are likely the result of microbiota-dependent differences in bile acid conversion. Indeed, the 7α-dehydroxylating intestinal bacterium *C. scindens* was associated with resistance to *C. difficile* infection (Buffie et al., 2015), and a simplified 12-species "oligo-mouse microbiota"(Oligo-MM^{12}) that represents the major murine intestinal bacterial phyla, but is 7α-dehydroxylation deficient, fails to confer resistance to CDI (Studer et al., 2016). Supplementation of the mice with *C. scindens* normalized the intestinal bile acid composition and impacted early colonization with *C. difficile*, but was insufficient to prevent pathogenesis (Studer et al., 2016). It is hypothesized that antibiotics might reduce the amount of secondary bile acids by eliminating bile acid converting bacteria, thereby increasing the propensity for CDI (Weingarden et al., 2014).

However, it is important to note that a model where primary bile acids strictly promote and secondary bile acids inhibit *C. difficile* germination and vegetation might be oversimplified. Physiological concentrations of the primary bile acids taurocholic acid, cholic acid and higher concentrations of chenodeoxycholic acid induced germination of spores, whereas physiological concentrations of chenodeoxycholic acid did not (Weingarden et al., 2016b) or even inhibited spore germination (Sorg and Sonenshein, 2009, 2010; Theriot and Young, 2015). The secondary bile acids deoxycholic acid and litocholic acid did not induce germination of spores (Weingarden et al., 2016b), in contrast to other studies which have shown that deoxycholic acid can be a germinant for *C. difficile* (Wilson, 1983; Sorg and Sonenshein, 2008).

Interestingly, recent work suggests that bile acids or bile acid-derived molecules may also influence toxin-dependent effects. In a small molecule screen, methyl cholate – but not cholate – was identified as a compound that inhibits auto-processing and receptor binding (Tam et al., 2015), leading to a reduction of toxin-dependent epithelial damage. Deoxycholate (0.02%) was found to inhibit toxin levels for some, but not all *C. difficile* strains *in vitro* (Thanissery et al., 2017).

Collectively, the data above suggest that bile acids may be an important component of an FMT (Weingarden et al., 2014) and that modulation of bile acid composition may be a viable therapeutic intervention (Buffie et al., 2015; Studer et al., 2016): FMT may introduce bacteria that affect bile acid composition and subsequently *C. difficile* germination and growth, or bile acids already present in the donor material may directly influence these processes. In support of the latter, it was reported that oral ursodeoxycholic acid (a secondary bile acid) was successfully used in conjunction with vancomycin to treat a patient with recurrent CDI pouchitis (Weingarden et al., 2016a). Both mechanisms, however, may not be mutually exclusive.

CARBOHYDRATES AND OTHER NUTRIENTS

Upon germination, vegetative cells use the nutrients available in the gut to proliferate. The nutritional requirements and capabilities of *C. difficile* have been extensively studied (Hafiz and Oakley, 1976; Karasawa et al., 1995; Scaria et al., 2014), but our understanding of the nutrient availability in the gut lumen in relation to *C. difficile* infection is limited. A metabolomic analysis showed that colonization with *C. difficile* leads to shifts in detectable metabolites and underscored the importance of amino acids and other nutrient availability for *C. difficile* colonization and pathogenesis (Fletcher et al., 2018).

In relation to CDI, some of the best characterized effects concern the liberation of sialic acid (**Figure 1C**). The mammalian host is protected from direct interaction with gut-dwelling microbes due to a physical barrier of heavily glycosylated mucus components produced by specialized cells in the intestinal mucosa. The mucus consists of an inner layer and a "loose" outer layer (Atuma et al., 2001). The outer layer can act as an important source of nutrition for bacteria capable of digesting its carbohydrate chains (Marcobal et al., 2013; Robinson et al., 2017). These carbohydrate chains are often capped with sialic acid, a 9-carbon-backbone monosaccharide that protects them from enzymatic reactions of exo- and endoglycosidases. Certain intestinal bacteria are able to remove sialic acids by expressing sialidases (Marcobal et al., 2011; Robinson et al., 2017). To protect sialic acids from these enzymatic actions they are modified through O-acetylation. This modification takes place in the inner mucus layer at the 7-O position of the sialic acid. As the mucus matures and moves to the outer layer the acetylation moves from the 7-O to the 9-O position. Some intestinal bacteria encode O-acetyl esterases that can remove the acetyl group, thus giving access to the sialidases that can remove the sialic acids. The O-acetyl esterases can

only remove 9-O- acetyl groups, but not 7-O- acetyl groups (Robinson et al., 2017). Thus, the inner mucus layer is always protected, whereas the outer layer can be a carbon source for intestinal bacteria. Not all bacterial species contain all enzymes required for digestion of complex carbohydrates. For example, *Bacteroides fragilis* encodes both sialidase and O-acetyl esterase activity, whereas *Bacteroides thetaiotaomicron* only has esterase activity. Bacteria that lack sialidases and esterases themselves can take advantage of the enzymatic activities provided by the other members of the microbiota to access nutrients and gain access to the epithelium (Marcobal et al., 2013; Robinson et al., 2017).

Antibiotic treatment can alter the balance in the mucolytic activities; elimination of species that efficiently scavenge sialic acid allows antibiotic-resistant pathogens to profit from the free sugars and expand rapidly as has been shown for both *Salmonella typhimurium* and *C. difficile* (Ng et al., 2013). In gnotobiotic mice colonized with sialidase-producing *B. thetaiotaomicron*, or by exogenous dietary administration of free sialic acid, a rapid expansion of the enteropathogens was observed. Colonization by a sialidase-deficient mutant of *B. thetaiotaomicron* reduced the levels of free sialic acid and impaired expansion of *C. difficile* (Ng et al., 2013). In an *in vitro* model it was also observed that other members of the microbiota compete more efficiently than *C. difficile* for monomeric glucose, N-acetylglucosamine and N-acetylneuraminic acid, in addition to sialic acid (Wilson and Perini, 1988).

Dietary carbon sources have also been implicated in *C. difficile* infection. Nosocomial outbreaks and sustained high infection rates in the community can frequently be attributed to the epidemic PCR ribotypes 027 and 078, respectively (Goorhuis et al., 2008; Bauer et al., 2011; He et al., 2013; Smits et al., 2016). Recently, it has been shown that both these PCR ribotypes can use low concentrations of trehalose more efficiently as a carbon source than non-epidemic types (Collins et al., 2018). In 027 strains this is the result of a point mutation in the trehalose repressor TreR that leads to stronger derepression in the presence of trehalose. Strains of PCR ribotype 078 appear to have acquired an extra gene cluster encoding a trehalose importer. The introduction of trehalose into the food chain might have preceded the expansion of the epidemic types, suggesting a causative effect (Collins et al., 2018).

Besides affecting growth, nutrients are also known to affect expression levels of the main virulence factors of *C. difficile*, the major clostridial toxins A (TcdA) and B (TcdB) (Bouillaut et al., 2015; Martin-Verstraete et al., 2016; Smits et al., 2016). For instance, the addition of glucose to *C. difficile* growth medium drastically reduces toxin gene expression (Dupuy and Sonenshein, 1998; Oliveira Paiva et al., 2016) and amino acids can both negatively and positively affect toxin levels (Yamakawa et al., 1994; Karlsson et al., 1999). In line with the effects observed for glucose, ethanolamine delays toxin production in *C. difficile*, in contrast to other gut pathogens that use ethanolamine as a virulence-inducing signal (Nawrocki et al., 2018). Ethanolamine is a breakdown product of membrane-derived phosphatidylethanolamine, which is abundant in the gut and is increased during inflammatory responses. Mutants of *C. difficile* that cannot utilize ethanolamine show enhanced virulence in a hamster model of infection (Nawrocki et al., 2018), that is exquisitely sensitive to levels of toxin A and B (Bakker et al., 2014).

It should be noted that most of these studies were performed *in vitro*, and it is largely unclear how the levels used in these experiments relate to luminal levels *in vivo*. Nevertheless, there is some recent evidence that dietary interventions can be relevant for CDI. Defined nutrient diets, in particular low protein diets, were found to increase survival of mice and reduce disease severity (Moore et al., 2015). Also, *C. difficile* burden in mice with humanized microbiota could be suppressed when the mice were fed with microbiota accessible carbohydrates found in dietary plant polysaccharides, whereas mice with diets deficient for such carbohydrates show a persistent infection (Hryckowian et al., 2018).

The effects of antimicrobials, nutrient availability, sensing nutritional status and toxin production by *C. difficile* come together in a recently formulated model (Hryckowian et al., 2017). It was suggested that *C. difficile* expansion following antibiotic treatment initially occurs without toxin production, possibly due to the presence of readily metabolizable nutrient sources such as glucose and sialic acid. After the expansion, *C. difficile* may switch to toxin production, leading to a favorable state of inflammation to inhibit competitors.

Considering the above, it is conceivable that the nutrient or carbohydrate availability in donor material affects the efficacy of FMT, either by affecting the (re)growth of *C. difficile* or by suppressing toxin expression and thereby reducing symptoms. However, these possible effects have not been systematically investigated to date and further studies are required to determine if nutrient variables should be monitored or adjusted in donor material.

SHORT AND MEDIUM CHAIN FATTY ACIDS

Above, we discussed the relevance of nutrient levels for the growth and pathogenesis of *C. difficile*. Next, we want to extend this, by focusing on a particular class of carbohydrates: the short and medium chain fatty acids - monocarboxylic acids with a carbohydrate chain length of 1 to 12 carbon atoms (Schonfeld and Wojtczak, 2016) (**Figure 1C**). These compounds have gained interest in the context of gastrointestinal disease due to their immune-modulatory and antimicrobial effects (Maslowski et al., 2009).

Short-chain fatty acids (SCFAs; chain length 1–6), are the main products of dietary fibers that are fermented by anaerobic gut bacteria and serve as substrates for energy metabolism (Roediger, 1980; den Besten et al., 2013). The most abundant SCFAs are acetate, propionate and butyrate (den Besten et al., 2013; Schonfeld and Wojtczak, 2016). Other common end-products of primary fermenters are the organic acids succinate and lactate, which are metabolized by other bacteria (Ferreyra et al., 2014). In mice, SCFAs bind G-protein coupled receptor 43 (GPR43) which results in resolution of inflammatory responses providing

a link between immune and inflammatory responses, diet and the metabolism of gut bacteria (Maslowski et al., 2009). Many SCFAs can be associated with an improvement of gut barrier function and also possess anti-inflammatory properties (Tedelind et al., 2007; Chen et al., 2017).

C. difficile metabolizes succinate to butyrate, particularly under antibiotic treatment or chemically induced diarrhea that results in an abundance of succinate and a reduction of acetate and butyrate (Ferreyra et al., 2014). A mutant *C. difficile,* deficient in succinate utilization, and therefore butyrate production, displays attenuated growth under both these conditions (Ferreyra et al., 2014). Though succinate is generally not detected in a healthy human gut, it was found that succinate accumulates in pigs suffering from antibiotic associated diarrhea (Tsukahara and Ushida, 2002). Succinate therefore may be depleted by cross-feeding (i.e., taken up and metabolized) and antibiotic use may perturb these interactions (Ferreyra et al., 2014).

Several studies have reported differences in SCFA producing bacteria and/or SCFA levels between healthy subjects and CDI patients: 16S rRNA gene analysis shows that butyrate producers, such as Ruminococcaceae and Lachnospiraceae families, are significantly reduced in CDI patients compared to controls (Antharam et al., 2013; Song et al., 2013; Ling et al., 2014; Lamendella et al., 2018). Concomitantly, increased levels of succinate, lactate, formate producers such as *Bacteroides* spp. were found (Antharam et al., 2013; Ling et al., 2014).

Short-chain fatty acid levels also appear to correlate with colonization resistance against *C. difficile* in certain cases. Mice treated for 2 weeks with cefoperazone are susceptible to colonization by *C. difficile* and metabolomics analyses showed reduced levels of acetate, propionate and butyrate compared to non-treated mice (Theriot et al., 2014). Six weeks after cessation of the antimicrobial treatment, colonization resistance was fully restored and SCFA levels recovered, though not to the levels observed in non-treated mice (Theriot et al., 2014). In earlier work, colonization resistance in hamsters was also found to coincide with high levels of SCFAs, that inhibit *C. difficile* growth (Rolfe, 1984). Cecal SCFA levels increase in hamsters from day 1, and reach a maximum at 19 days. Hamsters are susceptible to *C. difficile* colonization between days 4 and 15 and levels of SCFA corresponding to day 16 onward are bactericidal to *C. difficile* cultures *in vitro* (Rolfe, 1984).

The correlations between CDI and SCFAs have triggered investigations to modulate CDI development and progression through dietary interventions. Non-digestible oligosaccharides (dietary fiber) are carbohydrates resistant to the effects of gastrointestinal enzymes, but can be fermented to SCFAs by members of the colonic microbiota. Indeed, the addition of fiber to an *in vitro* model using fecal inoculums from pigs leads to high levels of acetate, propionate and butyrate that correlates with the expansion of anaerobic bacteria (May et al., 1994). In an *in vitro* model that simulates the conditions of the proximal part of the large intestine, the introduction of *C. difficile* leads to a suppression of propionate production and an increase in the branched-chain fatty acids isobutyrate and isovalerate (van Nuenen et al., 2003). The addition of inulin, a non-digestible oligosaccharide, reversed this effect and increased the total amount of SCFAs by 50% (van Nuenen et al., 2003). In another experiment, supplementation of *in vitro* cultures with different oligosaccharides increased SCFA production and did not result in detectable *C. difficile* toxin (Hopkins and Macfarlane, 2003). However, this was not the case during clindamycin treatment, where supplementation led to a reduction in SCFAs and conditions conducive for toxin production by *C. difficile*. (Hopkins and Macfarlane, 2003)

Despite the evidence presented above, a role for SCFA in control of CDI is not undisputed. No correlation was found between a qualitative SCFA profile of fecal emulsion and its ability to inhibit the growth of *C. difficile in vitro* (Borriello and Barclay, 1986). Furthermore, in a gnotobiotic mouse model, SCFAs alone did not affect colonization by *C. difficile* (Su et al., 1987). More recently, partial restoration of colonization resistance was reported when germ free mice were pre-colonized with *Lachnospiraceae*, but not with *E. coli* (Reeves et al., 2012). Though many *Lachnospiraceae* are SCFA producers (Scott et al., 2014), no association between SCFA production and *C. difficile* colonization levels was found (Reeves et al., 2012). Importantly, neither a fecal filtrate (containing SCFA), nor SCFAs were able to clear a *C. difficile* infection in mice (Lawley et al., 2012).

Medium chain fatty acids (MCFAs; with chain length 7–12) are predominantly derived from triglycerides and phospholipids that are ingested as part of plant oils and milk products (Schonfeld and Wojtczak, 2016). MCFAs can have antimicrobial activity, and modest activity of the 12-carbon lauric acid – a constituent of virgin coconut oil - against *C. difficile* has been reported (Shilling et al., 2013; Yang et al., 2017). Exposure of *C. difficile* cells to lauric acid leads to oxidative damage and cell lysis at higher concentrations (Shilling et al., 2013; Yang et al., 2017). Interestingly, orogastric pre-treatment with lauric acid decreased symptoms in a mouse model of CDI. However, at present it is unclear if these effects are due to an antimicrobial effect on *C. difficile* cells or due to an effect on the host (Yang et al., 2017).

In total, a causal relationship between SCFA and MCFA levels and *C. difficile* pathogenesis remains doubtful. Though a role for these compounds in donor material seems plausible, we expect that an analysis of fatty acid composition will potentially only be informative when placed in the context of the microbial composition and other variables.

BIOLOGICAL WARFARE

Other species of the microbial community not only interfere with *C. difficile* by competing for, or cross-feeding on, nutrients. Many bacterial species are known to produce antimicrobial compounds that allow the elimination of competitors (Tracanna et al., 2017). It is conceivable that the microbiota transferred by FMT produces specific bacteriocins that kill *C. difficile* (Khoruts and Sadowsky, 2016) (**Figure 1D**). Bacteriocins are proteinaceous antimicrobial compounds and are synthesized in many bacteria by ribosomes during translation (Le Lay et al., 2016). Their inhibitory activity may be small-spectrum or broad-spectrum and there are bactericidal and bacteriostatic bacteriocins. In general, their activity is directed against bacteria that are phylogenetically

close to the producing bacteria (Tagg et al., 1976). Some of the antimicrobial peptides that target *C. difficile* are discussed below.

Thuricin CD is a two-component bacteriocin, consisting of peptides Trn-α and Trn-β, produced by *Bacillus thuringiensis* DPC 6431, a bacterium derived from a human fecal sample (Rea et al., 2010; Sit et al., 2011). It displays a narrow spectrum of activity against mainly spore-forming gram-positive bacteria of the class Clostridia and Bacilli. All *C. difficile* isolates tested were sensitive to supernatants of *B. thuringiensis* DPC 6431, including the epidemic PCR ribotype 027 NAP1. In an *ex vivo* distal colon model, thuricin CD showed similar efficacy as metronidazole (Rea et al., 2010) and vancomycin (Rea et al., 2011).

Nisin is a bacteriocin with broad antimicrobial activity against a wide range of gram-positive bacteria (Lubelski et al., 2008). It can be isolated from the known gut residents such as *Blautia obeum* A2-162 (Hatziioanou et al., 2017) and species that can proliferate in conditions that resemble the gut, including *Lactococcus lactis* UL719 (Le Lay et al., 2015). *In vitro* studies show that nisin can inhibit spore germination and vegetative growth of *C. difficile* (Le Lay et al., 2016). In a human colon model, *L. lactis* UL719 did not significantly alter the microbiota composition and was not effective against *C. difficile*, likely because nisin produced *in vivo* does not reach inhibitory levels (Le Lay et al., 2015).

Lactobacillus reuteri carrying the *pocR* gene converts glycerol to reuterin, a broad-spectrum antimicrobial compound (Spinler et al., 2017). Reuterin induces oxidative stress, most likely by modifying thiol groups (Schaefer et al., 2010). Reuterin production increases in *L. reuteri* upon interaction with other bacteria (Schaefer et al., 2010). Co-delivery of *L. reuteri* and glycerol was effective and decreased the abundance of *C. difficile* relative to the bacterial load in fecal mini-bioreactor arrays pre-treated with antibiotics, without significantly affecting the microbial composition, whereas reuterin, *L. reuteri* or glycerol alone did not achieve this effect. This indicates that viable *L. reuteri*, substrate and active reuterin production is required for growth inhibition of *C. difficile* (Spinler et al., 2017).

Other antimicrobial compounds with activity against *C. difficile*, alone or in combination, include enterococcal durancin (Hanchi et al., 2017), formicin from *Bacillus paralicheniformis* (Collins et al., 2016), microbisporicin from the actinomycete *Microbispora* (Castiglione et al., 2008), and the lactococcal lacticin 3147 (Rea et al., 2007).

The potential for members of the microbiota to inhibit *C. difficile* is underscored by the fact that the clinically used therapeutic fidaxomicin, as well as the pre-clinical compound surotomycin were identified as natural products (Kociolek and Gerding, 2016). Fidaxomicin is produced by the actinomycete *Dactylosporangium aurantiacum* subspecies *hamdenesis* as a byproduct of fermentation. It prevents transcription by inhibiting bacterial RNA polymerase and is bactericidal against *C. difficile*. Fidaxomicin is non-inferior to oral vancomycin in clinical response and superior to oral vancomycin in reducing recurrent CDI (Louie et al., 2011; Cornely et al., 2012). Surotomycin, a cyclic lipopeptide antibiotic with a core derived from *Streptomyces roseosporus*, acts on the membrane stability of *C. difficile*, both in logarithmic and stationary phases (Knight-Connoni et al., 2016). It has a low oral absorption that allows high concentrations in the gastrointestinal tract to be achieved. Despite promising phase II results, phase III studies found the compound to be inferior to vancomycin (Boix et al., 2017). Both fidaxomicin and surotomycin claim a minimal impact on the gut microbiota (Kociolek and Gerding, 2016).

In addition to antimicrobial compounds produced by other members of the microbiota, the lumen of the colon also contains host-defense molecules that might play a role in CDI progression and pathogenesis (**Figure 1D**). These include defensins, cathelicidins such as LL-37 and lysozyme (McQuade et al., 2012). Defensins (α-defensins: HD5-6 and HNP1-4; β-defensins: HBD1-4) are small cationic peptides derived from intestinal Paneth cells, neutrophils or epithelial cells (Nuding et al., 2014). The human cathelicidin LL-37 is released upon cleavage of the precursor hCAP-18, which is produced in epithelial cells and some immune cells (Nuding et al., 2014). Lysozyme hydrolyses peptidoglycan bonds between N-acetyl-glucosamine and N-acetylmuramic acid and as a result the bacteria become sensitive to lysis (Chai et al., 2015).

Defensins and LL-37 were tested alone or in combination with antibiotics (tigecycline, moxifloxacin, piperacillin-tazobactam, and meropenem) against toxigenic and non-toxigenic *C. difficile* strains (Nuding et al., 2014). The antimicrobial peptides alone demonstrated some antimicrobial activity against *C. difficile*, but when combined, an additive effect was observed (Nuding et al., 2014). Certain treatments resulted in increased toxin release, presumably due to lysis of *C. difficile* (Nuding et al., 2014). This suggests that antimicrobial peptides alone or in combination with antibiotics not only exert a positive effect on CDI (clearance of *C. difficile*), but could potentially also worsen symptoms (by inducing more inflammation). Interestingly, α-defensins (HNP-1, HNP-3, and HD-5) appear to inhibit toxin B, but not toxin A, activity. The inhibition was dose dependent and reversible. β-defensins and LL-37 did not inhibit toxin A or toxin B (Giesemann et al., 2008). Similarly, LL-37, or the murine homolog cRAMP, can modulate the inflammatory response to toxins in a mouse model of infection (Hing et al., 2013). Lysozyme can act synergistically with bacterial antimicrobial peptides, as shown for nisin (Chai et al., 2015).

The effectiveness of human defense molecules might depend on the *C. difficile* isolate as variation in susceptibility to LL-37 was observed between *C. difficile* strains, with epidemic ribotype 027 strains being less susceptible than non-RT 027 strains (McQuade et al., 2012). *C. difficile* is capable of mounting a response to antimicrobial peptides and lysozyme (McBride and Sonenshein, 2011a,b; Hastie et al., 2014), but the contribution of these mechanisms to the observed variability is unknown.

There is very limited data on the role that defense molecules might play in FMT. A study describing the colonoscopic FMT for the treatment of CDI reported that LL-37 levels in plasma were significantly increased 3 months post-FMT, compared to pre-FMT and 3-weeks post-FMT (Konturek et al., 2016). As cathelicidins are important in preventing intestinal barrier dysfunction, this was taken as an indication of recovery from

CDI-damage (Konturek et al., 2016). It does indicate however, that healthy subjects likely demonstrate higher levels of LL-37 than CDI patients. It has been demonstrated that endogenous levels of cathelicidins are likely insufficient to maintain barrier function, but that high levels of exogenously provided LL-37 or cRAMP can reduce CDI symptoms in a mouse model (Hing et al., 2013).

Overall, we believe that antimicrobial peptides – whether derived from the microbiota or the host – have the potential to contribute to the efficacy of FMT. However, a clear correlation between FMT success and levels of these compounds in donor material remains to be established.

BACTERIOPHAGES

Above we discussed how inter-bacterial competition and host-defense can modulate the gut microbiota. However, another level of complexity exists in the form of bacteriophages (**Figure 1D**).

Bacteriophages are viruses, composed of proteins that encapsulate a DNA or RNA genome, and replicate within bacteria and archaea (Salmond and Fineran, 2015; Ofir and Sorek, 2018). Binding of bacteriophages to specific receptors on the bacterial cell determines their host range (Dowah and Clokie, 2018). Lytic phages infect, multiply in and lyse bacterial cells. In contrast, lysogenic phages can integrate into host DNA and replicate along with it, or become established as a plasmid without immediate lysis of the host (Salmond and Fineran, 2015; Ofir and Sorek, 2018). Phage exposure can not only affect the composition of the microbiota, but may also alter different phenotypes, including virulence and biofilm formation (Mirzaei and Maurice, 2017; Fernandez et al., 2018).

Prophages and phages have been found in pathogenic clostridia (Hargreaves and Clokie, 2014; Fortier, 2017). For instance, neurotoxin and α-toxin (TcnA) of *Clostridium botulinum* types C and D and *Clostridium novyi*, respectively, are encoded on prophages. In *C. difficile*, prophage carriage is high and one particular strain has been found to contain binary toxin on a prophage (Hargreaves et al., 2013; Fortier, 2017; Riedel et al., 2017). Phages known to infect *C. difficile* belong to the order of Caudovirales and, more specifically, the families Myoviridae and Siphoviridae (Hargreaves and Clokie, 2015; Ramirez-Vargas et al., 2018). As observed for other viruses, (pro)phages have been found to alter (virulence) gene expression in *C. difficile* (Goh et al., 2005; Govind et al., 2009; Sekulovic et al., 2011; Hargreaves et al., 2014b; Sekulovic and Fortier, 2015). CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) adaptive immune systems are widespread among bacteria and archaea. Recent studies have shown that these systems have minimal long-term evolutionary effects in limiting horizontal gene transfer (Pawluk et al., 2018). *C. difficile* contains a CRISPR-Cas system that provides defense against plasmid DNA and bacteriophages, as has been demonstrated in reference strain 630 and the epidemic ribotype 027 strain R20291 (Hargreaves et al., 2014a; Boudry et al., 2015; Andersen et al., 2016).

Phages are induced during CDI as sterile filtrates of CDI patients contained multiple phages (Meessen-Pinard et al., 2012). CDI patients display a higher abundance of Caudovirales than healthy household controls, and lower diversity, richness and evenness (Zuo et al., 2018). Moreover, treatment with antibiotics such as quinolones induced prophage mobility *in vitro* (Meessen-Pinard et al., 2012), as has also been observed by others (Modi et al., 2013). It therefore seems likely that both the virulence potential and horizontal gene transfer could be affected under conditions that sustain *C. difficile* expansion and pathogenesis.

Phages can be employed to treat bacterial infectious diseases (Abedon, 2017). At a time of growing antibiotic resistance in bacteria and the resulting restrictions on the use of antibiotics, bacteriophages can provide an alternative means of eliminating pathogens. Is it possible to use bacteriophages to treat CDI? Phage ΦCD27 demonstrates a significant reduction in *C. difficile* cell numbers and toxin production in an *in vitro* human colon model of CDI, without major effects on other members of the microbiota (Meader et al., 2010; Meader et al., 2013). Combinations of phage in batch cultures resulted in a 6-log reduction of *C. difficile* ribotype 014/020 (Nale et al., 2018), a type commonly identified amongst clinical isolates (Bauer et al., 2011), and were found to be able to penetrate established biofilms (Nale et al., 2016a). Though resistance to individual phages can be problematic, the resistant *C. difficile* remain susceptible to other phages (Nale et al., 2016b). Results obtained in animal models largely mirror the *in vitro* results. In hamsters, the administration of optimized phage cocktails resulted in reduced *C. difficile* colonization and recovery of free phage from the lumen of the cecum and the colon; moreover, pre-treatment with a phage cocktail delayed the onset of disease (Nale et al., 2016b). In a wax moth (*Galleria mellonella*) model, a phage cocktail was effective alone, or as adjunct therapy to vancomycin (Nale et al., 2016a). Besides live phages, phage-derived proteins are also explored as alternatives for treatment (Mayer et al., 2011; Kirk et al., 2017). Notably, phages demonstrate increased virulence toward bacteria in the presence of eukaryotic cells (Shan et al., 2018).

There are a limited number of studies that investigate phages during FMT treatment. A case study of a recurrent CDI patient demonstrated donor-similar characteristics of the virome following FMT (Broecker et al., 2017). Similarly, after FMT of a single healthy donor to three ulcerative colitis patients, donor-similar viral sequences were readily detected (Chehoud et al., 2016). Interestingly, temperate phages such as Siphoviridae were more likely to be transferred, suggesting that transfer or establishment may be dependent on lysogeny (Chehoud et al., 2016). An ultra-deep metagenomic sequencing study of viral transfer during FMT analyzed longitudinal samples of 9 CDI patients receiving FMT, and 5 receiving vancomycin treatment (Zuo et al., 2018). FMT treatment decreased the absolute abundance of Caudovirales, and treatment response correlated with the presence of donor-derived phage among those that were detected (Zuo et al., 2018). This suggests a role for Caudovirales bacteriophage in the efficacy of FMT. This is supported by a recent study where sterile (bacteria-free)

fecal filtrates were transferred to 5 patients with symptomatic chronic-relapsing CDI (Ott et al., 2017). The transfer of the filtrates, that showed a complex signature of bacteriophages, prevented recurrent CDI in all 5 patients for a minimum period of 6 months.

Taken together, it appears that the concept of CDI-predisposing dysbiosis, as a result of for instance antimicrobial treatment, could be extended to include bacteriophage and that phage therapy could be useful in the treatment of disease (Manrique et al., 2017).

OTHERS

So far, this review has focused on variables with a strong biotic component. However, abiotic factors, including metal availability, are known to affect infectious diseases (Fischer Walker and Black, 2004). Several secreted and surface-exposed proteins of *C. difficile* contain zinc as a co-factor, prompting an investigation of the effects of dietary zinc in a mouse model of CDI (Zackular et al., 2016). It was reported that mice on a high-zinc diet displayed a reduced diversity of their gut microbiota, and a concomitant reduction in threshold of antibiotics needed to induce CDI. Furthermore, consistent with a role in Toxin A auto-processing (Chumbler et al., 2016), CDI was exacerbated by an increased toxin activity in mice on a high-zinc diet. The mechanisms by which excess Zn alters the structure of the gut microbiota is unknown, but the authors postulate that it is a combination of several factors and consider metal toxicity to specific bacterial species as one of them (Zackular and Skaar, 2018). Human calprotectin is a zinc binding protein with broad activity against bacterial pathogens (Zackular et al., 2015). Consistent with a role in the innate immune response against *C. difficile*, calprotectin levels correlate with adverse outcome in CDI (Rao et al., 2016) and calprotectin-deficient mice show more severe CDI (Zackular et al., 2016).

Other metals have also been implicated in CDI. Divalent calcium ions are important for efficient germination of *C. difficile* and patients with inefficient calcium adsorption have a predisposition for CDI (Kochan et al., 2017).

Though the effects of levels of metals and metal-binding proteins such as calprotectin have not been systematically investigated, there is an intriguing possibility of modulating the efficacy of FMT by adjusting metal availability.

CONCLUSION

Many studies have shown that FMT is an effective treatment in patients with recurrent CDI (van Nood et al., 2013; Moayyedi et al., 2017; Quraishi et al., 2017; Terveer et al., 2017a; Ooijevaar et al., 2018). In the future, FMT may find other applications beyond *C. difficile* infections (Bakker and Nieuwdorp, 2017). However, since systematic investigations of FMT are relatively new, the mechanisms underlying the efficacy remain largely unknown. We expect that different mechanisms may play a role for other diseases than CDI.

We believe that it is unlikely that a single factor is responsible for the efficacy of FMT; the possible mechanisms examined in this review indicate that although individual variables evidently can modulate CDI, the mechanism of FMT is likely multifactorial. Indeed, FMT was found to restore both SCFA levels and bile acid metabolism (Seekatz et al., 2018). It should be noted that the gut microbiota is both influenced by, and influences, levels of compounds such as bile acids, short chain fatty acids and nutrients, complicating analyses of cause and effect (Seekatz and Young, 2018).

Current evidence suggests that part of the success of FMT can be attributed to the reconstitution of a robust and diverse microbiota of the gut (Lawley et al., 2012; van Nood et al., 2013). These findings were challenged, however, by the recent report that sterile filtrates were effective in treating recurrent CDI (Ott et al., 2017). It should be noted that these results have not been replicated yet in a randomized control trial, and are in contradiction with a study in mice that shows that transfer of buffer, fecal filtrate, a solution of short chain fatty acids or a single bacterial strain is unable to clear a *C. difficile* infection (Lawley et al., 2012). Perhaps non-bacterial components of a fecal filtrate are capable of transiently suppressing *C. difficile* growth, while allowing regrowth of a seeding population of a diverse microbiota that is still present in the patient's gut. The use of sterile fecal filtrates might be beneficial for certain patient groups.

Fecal microbiota transplantation leads to a fast improvement of symptoms and generally resolves CDI within days. An open question is whether such a short period is sufficient for stable engraftment of the transplanted microbial community in the host gut, with the concomitant eubiosis that is believed to suppress *C. difficile*. With regard to the mechanisms described in this review, such a fast improvement upon FMT appears most compatible with agents that directly affect *C. difficile* viability, possibly penetrating biofilms or pseudomembranes, such as antimicrobials or phage (Ott et al., 2017). Alternatively, it may be the result of direct suppression of inflammation, for instance through the action of defensins on toxin activity (Giesemann et al., 2008), or yet undefined compounds affecting host pathways underlying the inflammatory response (Cowardin and Petri, 2014; Chandrasekaran and Lacy, 2017). A crucial aspect missing from our current understanding of FMT is the role that host factors, both genetic and immunological, play in treatment efficacy.

We expect that comprehensive investigations – including the bacterial and bacteriophage composition, metabolites and small molecules – of donor material, as well as fecal contents of patients before and after FMT, will reveal how these are connected and will aid in the rational design of a synthetic donor infusion.

AUTHOR CONTRIBUTIONS

AB, ET, RZ, BH, JC, EK, and WKS performed a literature review. AB, JC, and WKS wrote the review. AB prepared the figure with

input from all authors. All authors read, edited, and approved the final text.

FUNDING

The Center for Microbiome Analyses and Therapeutics was supported by start-up funds from the Leiden University Medical Center. WKS was supported by a VIDI fellowship (grant number 864.13.003) VIDI of the Netherlands Organisation for Scientific Research and funds from the Leiden University Medical Center. BH, ET, and EK received funds from Vedanta Biosciences Inc. for microbiome related studies. The NDFB is supported by grant number 1708810011 from The Netherlands Organisation for Health Research and Development (ZonMW).

ACKNOWLEDGMENTS

ET and EK acknowledge the valuable input of the other members of the Netherlands Donor Feces Bank, Dr. J. Keller (LUMC), Dr. M. P. Bauer (LUMC), Dr. A. Goorhuis (Academic Medical Centre, Amsterdam), Dr. R. Ooijevaar (Free University Medical Center, Amsterdam), Dr. Y. van Beurden (Free University Medical Centre, Amsterdam), and Dr. E. van Nood (Erasmus Medical Centre, Rotterdam).

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Conflict of Interest Statement: ET and EK are part of the Netherlands Donor Feces Bank (NDFB). WKS has performed research for Cubist. EK has performed research for Cubist, Novartis and Qiagen, and has participated in advisory forums of Astellas, Optimer, Actelion, Pfizer, Sanofi Pasteur and Seres Therapeutics. BH, ET, and EK have received an unrestricted grant from Vedanta Biosciences Inc. The companies and the NDFB had no role writing this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 July 2018
doi: 10.3389/fmicb.2018.01701

Characterization of Bacteriophages Infecting Clinical Isolates of *Clostridium difficile*

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Clostridium difficile is recognized as a problematic pathogen, causing severe enteric diseases including antibiotic-associated diarrhea and pseudomembranous colitis. The emergence of antibiotic resistant *C. difficile* has driven a search for alternative anti-infection modalities. A promising strategy for controlling bacterial infection includes the use of bacteriophages and their gene products. Currently, knowledge of phages active against *C. difficile* is still relatively limited by the fact that the isolation of phages for this organism is a technically demanding method since bacterial host themselves are difficult to culture. To isolate and characterize phages specific to *C. difficile*, a genotoxic agent, mitomycin C, was used to induce temperate phages from 12 clinical isolates of *C. difficile*. Five temperate phages consisting of ΦHR24, ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50 were successfully induced and isolated. Spotting assays were performed against a panel of 92 *C. difficile* isolates to screen for susceptible bacterial hosts. The results revealed that all the *C. difficile* phages obtained in this work displayed a relatively narrow host range of 0–6.5% of the tested isolates. Electron microscopic characterization revealed that all isolated phages contained an icosahedral head connected to a long contractile tail, suggesting that they belonged to the *Myoviridae* family. Restriction enzyme analysis indicated that these phages possess unique double-stranded DNA genome. Further electron microscopic characterization revealed that the ΦHN10 absorbed to the bacterial surface via attachment to cell wall, potentially interacting with S-layer protein. Bacteriophages isolated from this study could lead to development of novel therapeutic agents and detection strategies for *C. difficile*.

Keywords: ***Clostridium difficile*, temperate phage, phage induction, host range, *Myoviridae***

OPEN ACCESS

Edited by:
Peter Mullany,
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United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *01 March 2018*
Accepted: *09 July 2018*
Published: *31 July 2018*

Citation:
Phothichaisri W, Ounjai P, Phetruen T, Janvilisri T, Khunrae P, Singhakaew S, Wangroongsarb P and Chankhamhaengdecha S (2018) Characterization of Bacteriophages Infecting Clinical Isolates of Clostridium difficile. Front. Microbiol. 9:1701. doi: 10.3389/fmicb.2018.01701

INTRODUCTION

Antimicrobial resistance has become one of the most serious global healthcare problems. The world mortality rate from drug resistant bacteria is estimated to be 700,000 per year and if these trends continue, by 2050, 10 million deaths a year will be reached (O'Neill and The Review on Antimicrobial Resistance, 2014). A Gram-positive, spore-forming bacterium, *C. difficile* is considered one of the most important drug resistant pathogens (Harnvoravongchai et al., 2017)

and has been listed by the Centers for Disease Control and Prevention as an urgent threat. This anaerobic, toxin-producing bacterium is the leading cause of antibiotic-associated diarrhea in nosocomial setting (Rineh et al., 2014). *C. difficile* infection (CDI) can result in a wide range of clinical symptoms from asymptomatic carrier, mild diarrhea with low fever, colitis, pseudomembranous colitis to life-threatening fulminant colitis (Libby and Bearman, 2009; Buffie et al., 2012). Current treatment options for CDI largely depend on specific antibiotic treatment combined with supportive care, which address the high rate of recurrence due to resistant trait development (Hedge et al., 2008). The most recent option for recurrent CDI treatment is fecal microbiota transplantation (FMT), by using stool collected from healthy donors to treat CDI patients. This treatment succeeds to treat dysbiosis patients with remarkable high efficacy rate up to 81% (Drekonja et al., 2015). Not only bacteria but also bacteriophages and other active components are transferred in FMT that can affect the treatment outcome (Zuo et al., 2017). Therefore, the screening and validation of stool FMT donors are challenging for this approach. However, the incidence, severity and recurrence rates of CDI have markedly risen in both hospital and community settings over the past two decades (Vindigni and Surawicz, 2015). Declining of CDI was reported in last few years, which is considered due to good control, management, and following to CDI prevention guideline (Evans et al., 2017). The change in *C. difficile* epidemiology may be caused by inappropriate antibiotic usage, emergence of hypervirulent strains and outbreaks of CDI in hospitals (Aslam et al., 2005). Therefore, to mitigate the severity of CDI outbreaks and reduce the disease recurrence, alternative approaches for effective control of CDI are urgently needed (Joerger, 2003; Rea et al., 2013).

Bacteriophages pose one of the promising alternatives to be used in a control of resistant bacteria (Lin et al., 2017). Phages can be highly specific to their bacterial hosts. Many of the phages exhibit a very narrow host range. They can target, degrade and penetrate into bacterial biofilm and form plaques (Parasion et al., 2014; Nale et al., 2016). These characteristics highlight their potentials to be utilized as novel therapeutics for antibiotic resistant bacterial infections (Viertel et al., 2014). The possibility of using phages for curing bacterial infections was first described by Felix d' Herelle since the early 1900's (Wilkinson, 2001). However, after the advent of antibiotics in the 1940s, less attention was paid to research in phage therapy (Abedon et al., 2011; Viertel et al., 2014; Lin et al., 2017). The interest in utilizing phages as antibacterial agents has recently re-emerged due to rapid increase in antibiotic resistance and decline in novel antibiotic discovery (Ventola, 2015; Lin et al., 2017). Recent advances in molecular and sequencing techniques opens a tremendous opportunity to engineer phages and phage-derived proteins for therapeutic applications.

Phages may adopt two alternative life cycles, which are lytic or lysogenic. The lytic phages kill the host bacteria by causing cell lysis, while the temperate phages are able to insert their genome into the host in their lysogenic life cycle, forming prophages and replicate with the host cell. However, temperate phages often switch back to lytic life cycle and break out from the host under stress condition (Lamont et al., 1993; Orlova, 2012). Previous reports have shown phages specific to *C. difficile* from environmental and clinical samples and demonstrated that all of the isolated phages did not follow a strictly lytic lifestyle, despite their lytic activity, as their genomes encode integrases (Goh et al., 2005; Govind et al., 2006; Meessen-Pinard et al., 2012; Sekulovic et al., 2014).

The interaction of bacteriophage and host is always initiated through the docking of phage particle to the corresponding receptors on host surface. Many components found on the bacterial surface were proposed to be phage receptors, including cell wall teichoic acid, cell surface glycoprotein, S-layer proteins, pili, and lipopolysaccharides (Larson et al., 1978; Rakhuba et al., 2010). Due to limited resources on *C. difficile* phages, the receptor of *C. difficile* phages has not yet been identified.

In this work, we isolated and established a library of phages from clinical *C. difficile* isolates. The morphology, host range analysis and pattern of their genetic materials of the *C. difficile* phages were characterized. Further investigations on the interaction between *C. difficile* and ΦHN10 phage suggested that S-layer protein could serve as a potential receptor of this phage in this bacterium. A library of phages established in this work could potentially provide fruitful information that could lead to further development of alternative treatment and detection for CDI.

MATERIALS AND METHODS

Bacterial Cell Culture and Growth Condition

Seventy-three clinical *C. difficile* isolates were used for prophage carriage determination and phage isolation. Twenty-six of them were previously isolated from *C. difficile* toxin-positive fecal samples of diarrheal patients admitted to Ramathibodi Hospital, Thailand, during 2010–2011 (Chankhamhaengdecha et al., 2013), and additional 47 strains were obtained from the National Institute of Health (NIH), Thailand. The isolates have previously been identified into 16 PCR ribotypes using agarose gel-based PCR ribotyping (**Table 1**). Nineteen *C. difficile* reference ribotypes used in this study were kindly provided by Prof. Nigel Minton, University of Nottingham. All isolates were routinely grown in pre-reduced brain heart infusion broth (BHI) supplemented with 1% sodium taurocholate. Cultures were incubated at 37°C in an anaerobic chamber (10% H_2, 10% CO_2, and 80% N_2) (Don Whitley Scientific, West Yorkshire, UK).

Identification of Lysogenic Strain From Clinical *C. difficile* Isolates

The presence of lysogenic strains in the clinical *C. difficile* strain collection was determined by PCR analysis. Two sets of degenerate primers targeting the *C. difficile* phage myovirus and siphovirus *holins* and the PCR amplification program were performed as described previously (Shan et al., 2012). Genomic DNA was extracted and purified from 73 clinical isolates of *C. difficile* using the E.Z.N.A. Bacterial DNA kit (Omega Bio-tek, GA, USA). The PCR products were analyzed through

TABLE 1 | *C. difficile* strains used in this work and prophage content.

Strain[a]	PCR ribotype[b]	PCR amplification of phage genes in group:		Induction with mitomycin C		Origin	Strain[a]	PCR ribotype[b]	PCR amplification of phage genes in group:		Induction with mitomycin C		Origin
		Myo	Sipho	Myo	Sipho				Myo	Sipho	Myo	Sipho	
Std001	001	+	–	ND		Reference Strains	HN1	NT-15	–	–	–	–	Clinically relevant strains, NIH
Std017	017	+	–				HN2	017	+	–	–	–	
Std020	020	+	–				HN3	017	+	–	–	–	
Std023	023	+	–				HN4	017	+	–	–	–	
Std027	027	–	–				HN5	017	+	–	–	–	
Std029	029	+	–				HN6	017	+	–	–	–	
Std046	046	+	–				HN7	017	+	–	–	–	
Std053	053	+	–				HN8	NT-16	+	+	–	+	
Std056	056	+	+				HN9	017	+	–	–	–	
Std070	070	–	+				HN10	017	+	+	–	+	
Std075	075	+	–				HN11	017	+	–	–	–	
Std077	077	+	–				HN13	NT-16	+	+	–	+	
Std081	081	+	–				HN16	017	+	+	+	+	
Std095	095	+	+				HN17	NT-16	+	+	–	–	
Std117	117	+	–				HN18	NT-16	+	+	–	–	
Std126	126	–	–				HN19	017	+	–	–	–	
Std131	131	+	+				HN20	017	+	–	–	–	
R20291	027	+	–				HN21	017	+	+	-	+	
630	012	+	–				HN22	NT-15	+	–	–	–	
HR1	NT-1	–	–	–	–	Faecal samples of diarrheal patients, Ramathibodi Hospital	HN23	NT-15	+	–	–	–	
HR2	NT-2	+	+	+	+		HN24	NT-15	–	–	–	–	
HR13	NT-11	+	–	+	–		HN25	NT-15	+	–	–	–	
HR24	NT-7	+	–	+	–		HN26	NT-15	+	–	–	–	
HR27	NT-7	+	+	–	–		HN27	NT-15	+	–	–	–	
HR29	NT-11	–	+	–	+		HN28	NT-15	+	+	–	–	
HR31	NT-6	+	–	+	–		HN29	NT-15	+	+	–	–	
HR37	NT-12	+	+	+	–		HN30	NT-15	+	+	+	–	
HR38	NT-3	+	–	–	–		HN31	NT-15	+	–	+	–	
HR39	020	+	–	–	–		HN32	NT-15	+	–	–	–	
HR44	017	+	+	+	–		HN33	NT-15	+	–	+	–	
HR49	017	+	+	–	–		HN34	NT-17	+	–	–	–	
HR58	NT-8	–	–	–	–		HN35	NT-17	+	–	–	–	
HR59	NT-13	+	–	–	–		HN36	NT-15	+	–	+	–	
HR65	NT-9	–	–	–	–		HN37	NT-17	+	–	-	–	
HR67	NT-10	+	–	–	–		HN38	NT-17	+	–	+	–	
HR74	NT-4	+	+	–	–		HN39	NT-17	+	–	+	–	
HR91	NT-14	+	–	–	–		HN40	NT-15	+	–	–	–	
HR98	017	+	–	–	–		HN41	NT-17	+	–	–	–	
HR118	017	+	–	–	–		HN42	NT-17	+	–	–	–	
HR136	017	+	–	–	–		HN43	NT-17	+	–	–	–	
HR156	NT-5	-	–	–	–		HN44	NT-17	+	–	–	–	
HR163	017	+	–	–	–		HN45	NT-17	+	–	–	–	
HR166	NT-11	–	+	–	–		HN46	NT-17	+	–	–	–	
HR184	017	+	+	+	–		HN47	NT-17	+	–	–	–	
HR376	NT-3	–	–	–	–		HN48	NT-17	+	–	–	–	
							HN49	NT-17	+	–	–	–	
							HN50	017	+	+	+	+	

[a,b]*Data of strain isolation and PCR ribotyping are included in the accompanying paper (S. Wongkuna, N. Malaisree, A. Aroonnual, T. Janvilisri, D. Chotiprasitsakul, P. Chongtrakool, P. Wangroongsarb, S. Chankhamhaengdecha, submitted for publication). ND, not determined; NT, ribotype patterns not related to the standard ribotypes.*

electrophoresis in 1.5% (w/v) agarose gels, stained with SYBR safe (Invitrogen), and visualized under UV radiation. The PCR products from 5 distinct *C. difficile* strains that were positive according to the amplification with these two sets of degenerate primers were sequenced to confirm that the amplified sequences were encoded by *C. difficile* phage *holins*.

High-Throughput Induction and Screening of Prophages From Lysogens

All lysogenic *C. difficile* strains were induced with mitomycin C using a high-throughput induction method. The method was modified from a previous protocol for rapid prophage induction from *Escherichia coli* (McDonald et al., 2010). Briefly, a 96-well plate containing BHI medium was inoculated with a single colony of lysogen and incubated at 37°C overnight under anaerobic condition. For phage induction, 10-fold dilution of overnight culture of each isolate in BHI medium was made into a fresh 96-well plate and incubated at 37°C for 8 h. Induction of phage was performed by adding 3 μg/ml mitomycin C to the culture with further incubation at 37°C for 12 h. Subsequently, the culture was diluted 10-fold. Phages were isolated using filtration of lysate through 0.22 μm filter (Merck Millipore, Germany). Isolated phages were then transferred into another 96-well plate. To get rid of host genomic DNA from the lysate, 5 μg/ml of DNaseI was then added to each well and incubated at 37°C for 1 h. The reaction was heat-inactivated at 99°C for 10 min. The completeness of digestion reaction was evaluated by PCR using bacterial *16s rRNA* gene specific primers, UFUL (5′-GCCTAACACATGCAAGTCGA-3′) and URUL (5′-CGTATTACCGCGGCTGCTG G-3′; (Khan et al., 2001)). The inducible temperate phage DNA in each heated lysates was confirmed by PCR using specific phage primers as described previously (Shan et al., 2012).

Phage Preparation

The strains of *C. difficile* containing inducible temperate phages, including HR24, HN10, HN16, and HN50, were chosen for large scale phage induction. Mitomycin C induction was performed on 500 ml of log phase *C. difficile* cultured in BHI broth supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ under anaerobic condition. After 12 h induction, lysates were centrifuged at 12,000 × *g* for 30 min and filtered through 0.45 μm filter membrane (Merck Millipore). Phage lysates were concentrated using polyethylene glycol (PEG) precipitation as previously described (Sambrook and Russell, 2001). Briefly, the phage lysates were salted out in 1 M NaCl. After centrifugation at 12,000 × *g* for 10 min at 4°C, 10% PEG-8000/$MgSO_4$ solution was added to supernatant fraction and incubated overnight. The samples were then pelleted down for 30 min at 12,000 × *g*. Pellets were dissolved in SM buffer (150 mM NaCl, Tris-HCl pH6.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$). Equal volume of chloroform was then added to the phage solution and was centrifuged at 12,000 × *g* for 5 min to induce phase transition. The upper phase was collected. The concentrated phage was then screened for its sensitive host using spot test (Beck et al., 2009) on a panel of 92 different *C. difficile* isolates. The lawns of the indicator hosts were independently created by mixing *C. difficile* culture with 3 ml of 0.6% molten TYs agar (3% tryptose, 2% yeast extract) supplemented with 10 mM $CaCl_2$ and 10 mM $MgCl_2$. The mixture was immediately overlaid on 1.5% BHI agar. The overlay was allowed to set for 5 min. After that, 10 μl of concentrated phage was then spotted onto the overlay of different *C. difficile* isolates. Following the incubation at 37°C overnight, the sensitive host of each phage was determined based on the plaque formation.

To further isolate a single strain of phage, the double agar layer method was used (Gencay et al., 2017). Briefly, 1.5 ml of *C. difficile* sensitive host was mixed with 0.1 ml of serially diluted phage solution and incubated at 37°C for 10 min. The mixture was subsequently transferred to 3 ml of 0.6% molten TYs (top). After that, the mixture was immediately overlaid on the preformed 1.5% BHI agar (bottom). The plate was allowed to sit at room temperature to let the soft agar in the top layer solidify. The plate was then incubated at 37°C overnight under anaerobic condition and was monitored for plaque formation. Single plaque of different sizes was collected and resuspended in SM buffer. Double agar layer method was repeated at least three times using the collected plaque suspension from the previous round until a single plaque morphology was achieved. The purified phage from the final round was then propagated in its sensitive host using liquid culture. Briefly, the sensitive host of each phage was grown in 100 ml of TYs broth at 37°C for 8 h under anaerobic condition. Subsequently, 1 ml of isolated phage lysate was added to the culture and incubated for 10 min, following by the addition of 100 ml of fresh TYs broth to the mixture. Following the overnight incubation, phage lysate was collected, filtered, and concentrated using PEG precipitation as described above. The enriched phage fraction was then further purified using CsCl gradient as previously described (Sambrook and Russell, 2001). Solid CsCl was added to phage solution to a final concentration of 0.75 g/ml. The mixture was centrifuged at 150,000 × *g* for 24 h at 4°C. The white-gray band representing phage particles were collected and dialyzed three times against 1 L of SM buffer at 4°C using 50 kDa dialysis tube (Spectrumlab, USA). The purified phage suspension was kept at 4°C for further experiment. In case of long-term phage storage, purified phage suspension was mixed with 7% (v/v) dimethyl sulfoxide (DMSO) and stored at −80°C.

Transmission Electron Microscopy (TEM) and Image Analysis

A droplet of selected *C. difficile* phages including ΦHN10, ΦHN16-1, ΦHN16-2, ΦHN50, and ΦHR24 were deposited on a continuous formvar-carbon or formvar-coated air glow discharged grids. The specimen was negatively stained using 2% uranyl acetate prior visualization under Tecnai T20 G^2 electron microscope operating at the accelerating voltage of 120 keV. Images were captured using a CCD camera (Gatan). Negative stain EM of *C. difficile* cells were also performed using 2% uranyl acetate (Ackermann, 2009). The images were visualized in a Tecnai T20 G^2 electron microscope at 1–1.5 μm defocus and recorded using Gatan CCD camera. Image analysis and Fourier calculation were conducted in Digital Micrograph software.

Host Range Determination

The host range of phages, ΦHN10, ΦHN16-1, ΦHN16-2, ΦHN50, and ΦHR24, was preliminary determined by spot test (Beck et al., 2009) on 92 different *C. difficile* isolates as described above. Positive spot tests were further confirmed by modified double agar layer method (Kropinski et al., 2009). One point five ml of *C. difficile* culture were mixed with 0.1 ml of serially diluted purified phage and incubated at 37°C for 10 min. The mixture was subsequently transferred to 3 ml of 0.6% molten TYs and immediately overlaid on the preformed 1.5% BHI agar and allowed soft agar in the top layer to solidify. The plate was then incubated at 37°C overnight under anaerobic condition. The plate was observed and the size and appearance of the plaques were noted. Plaque size was measure using Vernier caliper. Phage titer was calculated from the number of plaque formation.

Evaluation of Phage Stability

Stability tests were performed on 4 isolated phages (ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50, excluding ΦHR24 due to lack of its sensitive host). To evaluate the ability of phages to tolerate different pH conditions, isolated phage samples were assayed in TYs broth prepared at different pH, ranging from pH 2 to pH 11. The samples were incubated at 37°C for 3 h (Capra et al., 2006). After that, phage titers were determined using spot-titer assays. Ten-fold dilutions of phage (10^5-10^{10} PFU/ml) were spotted on lawn of bacterial host to determine phage existence. To examine thermal stability of isolated phages, samples of phages were incubated at various temperatures in a water bath (25, 37, 50, 60, and 70°C). The samples were collected at 5, 30, 60, 120, and 180 min (Capra et al., 2004). The titers of phages at various conditions were enumerated using spot-titer assays (Beck et al., 2009). The 10 μl volumes of several sample dilutions were spotted on a single plate, incubating, and observing for plaques.

Nucleic Acid Extraction and Restriction Profile Analysis

Nucleic acids of the 4 isolated phages including ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50 were extracted using modified phenol/chloroform method as described elsewhere (Moineau et al., 1994). Briefly, 3 ml of concentrated phages were treated with 50 μg/ml DNaseI and 50 μg/ml RNaseA (Sigma-Aldrich, UK) for 1 h at 37°C. The reaction was terminated by adding 0.5 volume of an SDS mixture (0.5 M Tris-HCl pH 9.0, 0.25 M EDTA, 2.5% SDS) and incubating at 65°C for 15 min. An equal volume of 25:24:1 phenol-chloroform-isoamyl alcohol (Merck, UK) was then added into the solution. The mixture was then vigorously mixed and centrifuged at 12,000 × *g* for 15 min at room temperature. The aqueous phase was collected. This phenol-chloroform-isoamyl alcohol extraction step was repeated for two times. Nucleic acids were then precipitated in ice-cold 70% ethanol. The pellet was resuspended in 50 μl of TE buffer pH 8 and stored at −20°C until use. The profiling of phage DNA was performed by treating isolated phage DNA with 12 restriction endonucleases including *Alu*I, *Dpn*I, *Acc*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hinc*II, *Hin*dIII, *Nde*I, *Nco*I, and *Pst*I, according to manufacturer's instructions. Restriction pattern were visualized using 1% agarose gel electrophoresis at 90 V for 1 h.

Phage Attachment and Phage Receptor Prediction

Cells in 5 ml of late log phase culture of *C. difficile* HN2, sensitive host of ΦHN10 phage, were collected by centrifugation at 3,000 × *g* for 5 min. Defective bacteria lacking of pili and flagella and other cellular appendages were prepared using mechanical agitation method (Novotny et al., 1969). The cells were resuspended in 200 μl phosphate buffer saline (PBS) supplemented with 10 mM $CaCl_2$, 10 mM $MgCl_2$, thioglycolic acid (TGA), and 5% sucrose. To disrupt cellular projection including pili and flagellum, bacterial cell suspensions were vigorously agitated in a vortex mixer and then subjected to incubation in a sonication bath at room temperature for 20 min. Defective bacterial cells lacking pili and other cellular appendages were then isolated by centrifugation at 5,000 × *g* for 2 min. Pellets of defective bacteria were resuspended in 200 μl fresh BHI broth. Susceptibility of intact and defective *C. difficile* cells to ΦHN10 was characterized by a spot-titer assay (Beck et al., 2009). To determine phage titer that is susceptible to each host cell types, 10-fold serial dilutions of ΦHN10 phage were prepared in culture media from 10^5 to 10^{10} PFU/ml. Then, 10 μl of each dilution of the phage were spotted on the double agar layer. The lowest phage concentration was quantified on the basis of the numbers of plaques. The putative phage receptor on bacterial host cell for phage binding was investigated using transmission electron microscope. Fourier transforms of the *C. difficile* S-layers were calculated using Gatan Digital Micrograph Software.

Extraction of S-Layer Proteins

S-layer proteins (SLPs) was extracted from *C. difficile* as previously described (Calabi et al., 2001). Overnight culture of *C. difficile* HN2 was transferred to 50 ml fresh BHI medium and cultured until OD_{600} reach 0.5. Cells were harvested by centrifugation at 3,000 × *g* for 20 min. Then cells were washed once with PBS buffer and resuspended in 200 μl of 200 mM glycine pH 2.2. Cells were incubated at room temperature for 30 min before removing intact and cell debris by centrifugation at 16,000 × *g* for 15 min at 4°C. S-layer protein-containing supernatant was collected, the pH of the solution was adjusted to 7 with 2 M Tris-HCl pH 9 and stored at 4°C.

Gel Shift Assay of S-Layer Protein

To investigate the interaction between isolated phage, ΦHN10 and S-layer proteins, 1 mg/ml of S-layer protein was preincubated with 0.1 mg/ml of ΦHN10 in PBS buffer containing 150 mM NaCl for 6 h. The mixture was then subjected to native-PAGE analysis using the running buffer consisted of 40 mM Tris pH 10, 40 mM acetate and 1 mM EDTA. The gel was stained with Coomassie to observe the shift of the S-layer protein.

RESULTS

Prophage Induction and Phage Morphology

Lysogenic strains within clinical isolates of *C. difficile* were determined by PCR targeting-holin genes of myovirus and siphovirus (**Supplementary Figure 1**). The PCR products were confirmed by DNA sequencing, which were further blasted against nucleotide database in National Center for Biotechnology Information (NCBI). The results confirmed that the amplified PCR products were *holin* genes of myovirus or siphovirus. The results revealed that 66/73 clinical *C. difficile* isolates were lysogenics. The inducibility in all of the 66 lysogenic strains was then investigated by mitomycin C using high-throughput induction and screening method. Twenty-two (32.8%) of temperate phages were mitomycin C inducible. Myovirus *holin* genes were found in the phage preparation of 15 bacterial isolates including HR13, HR24, HR31, HR37, HR44, HR184, HN10, HN23, HN30, HN31, HN33, HN36, HN38, HN39, and HN47. While 4 isolates including HR29, HN8, HN13, and HN21 were siphovirus positive and 3 isolates, HR2, HN16, and HN50, appeared to harbor both myo- and siphovirus as summarized in **Table 1**.

In this study, phages from 4 *C. difficile* isolates HN10, HN16, HN50, and HR24 were selected for further analyses. The phages induced from these isolates were assigned according to the names of their respective hosts as ΦHN10, ΦHN16, ΦHN50, and ΦHR24. Interestingly, two prophages with different sizes and morphological features could be induced from the strain HN16. These two phages were isolated against their sensitive host using double agar layer method. The phages were designated as ΦHN16-1 and ΦHN16-2 as shown in **Table 2**. All inducible temperate phages possessed long, non-flexible, and contractile tail, indicating that they are members of *Myoviridae* family, in the order of *Caudovirales*. The phages contained icosahedral capsid head with the size ranging from 45 to 70 nm in diameter and the contractile tail with the length ranging from 125 to 250 nm. In addition, phage tail-like particle structures (PT-LPs) were also observed in the lysate of the isolates HR37 and HN47. The overall structure appeared similar to a regular phage tail of myovirus, but without icosahedral head. The size of particle was ∼100 nm long, 20 nm wide with 40 nm horizontal cap at the one end (**Table 2**).

Host Range Determination

In our study, the host ranges of the 5 *C. difficile* phages including ΦHN10, ΦHN16-1, ΦHN16-2, ΦHN50, and ΦHR24 were preliminary determined against a panel of 92 *C. difficile* strains containing standard ribotypes and clinical isolates by spot test. However, this test has been demonstrated to over-estimate the host range of a phage (Mirzaei and Nilsson, 2015). Therefore, sensitive hosts were confirmed using double agar layer method. Although no susceptible host was found for ΦHR24. The other 4 temperate *C. difficile* phages displayed a narrow host range against panel of indicator hosts tested. ΦHN10 produced clear plaques on 6 different isolates including 630, HR118, HN2, HN6, HN9, and HN21. The 630 strain is ribotype 012 whereas the remainder were ribotype 017. ΦHN16-1, ΦHN16-2, and ΦHN50, could only lyse HN21, which was ribotype 017 (**Table 2**).

Stability of Phages at Different PH and Temperature

The pH stability of phage was carried out at different pH range from pH 2 to pH 11. After 180 min incubation, phages in this study were shown to have stable infectivity at wide ranges of pH from pH 5 to pH 10. While, the phages ΦHN10, ΦHN16-1, and ΦHN50 completely lost their infectivity at pH 2–4, ΦHN16-2 still maintained their activity at pH 4. Most of the 4 phages appeared to lose lytic activity at pH 11, except ΦHN50, which a significant decrease in activity was observed (**Figure 1**). The phage thermal tolerance was tested showing that ΦHN16-1, ΦHN16-2, and ΦHN50 had stable infection after incubation at 25, 37, and 50°C for 180 min and the phage infectivity was dramatically decreased at 60°C. More than 50% of ΦHN10 was still active after incubation at 50 and 60°C for 180 min. At 70°C, all phage particles lose their infectivity (**Figure 1**).

Genomic Restriction Profile

The DNA restriction profiles of 4 phages including ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50, were examined. They exhibited distinct DNA restriction patterns following *Hin*dIII digestion, as shown in **Figure 2**. Thus, all the 4 isolated phages are considered different phages.

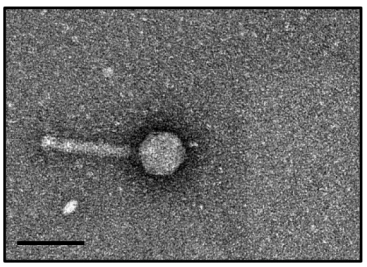
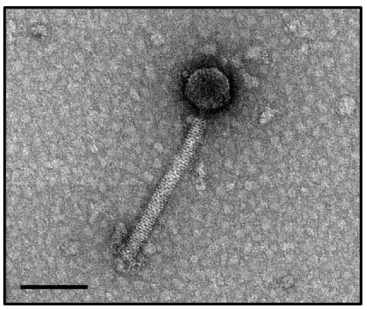
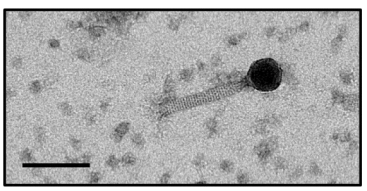
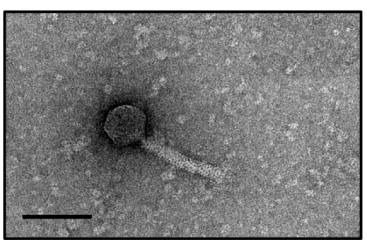
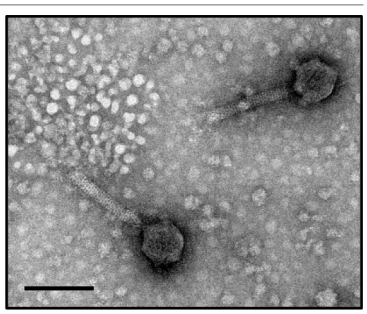
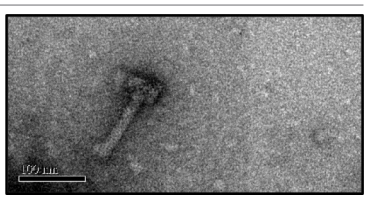
Phage Attachment on Bacterial Host

To investigate the potential phage receptor on bacterial cells, mechanical force was used to disrupt pili and part of bacterial cell surface from *C. difficile* HN2. It showed lower susceptibility to phage infection after mechanical treatment. In normal condition, HN2 host could be lysed by phage at 10^5 PFU/ml, while defective HN2 host could be lysed by higher concentration of phage at 10^9 PFU/ml (**Figure 3**). The results suggested that ΦHN10, as a representative *Myoviridae* phage, exhibited lower efficiency in infection to their defective host. Identification of putative phage receptor was performed using TEM analysis. Normal *C. difficile* HN2 cells possessed pili (**Figure 4A**), whereas the pili are absent in the mechanically treated cells (**Figure 4B**). Moreover, the treated cells appear to have thinner cell wall when compared with the untreated cells. Despite, having no pili, abundant binding of phages was observed on the cells of treated bacteria. The particle appeared to bind to a mesh-like structure on bacterial cell surface (**Figure 4C**). To determine the pattern of surface array, the phage binding areas in the images were subjected to Fourier analysis. The Fourier image revealed tetrahedral arrangement of the subunits (**Figure 4D**), suggesting that the mesh-like structure on *C. difficile* that binds to the phages is likely to be an S-layer.

Identification of Phage-Receptor

In order to confirm the interaction between S-layer proteins and ΦHN10 phage, S-layer proteins were isolated from its susceptible host, *C. difficile* strain HN2. Analysis of the S-layer-phages interaction on the native-PAGE showed that the migration of S-layer proteins become retarded after incubating with phages when compared with the S-layer proteins alone

TABLE 2 | Morphological characteristic and specific host of isolated phage in the family of Myoviridae.

Phage	Specific host	Plaque appearance, diameter (mm)	Capsid (nm)	Tail length/tail width (nm)	Phage morphology
ϕHR24	None	None	∼70	∼140/20	
ϕHN10	630, HR118, HN2, HN6, HN9, HN21	Clear, 1.1[a]	∼70	∼250/23	
ϕHN16-1	HN21	Clear, 0.6	∼45	∼130/15	
ϕHN16-2	HN21	Clear, 1.1	∼60	∼125/20	
ϕHN50	HN21	Clear, 0.7	∼60	∼135/22	
PT-LP	None	None	None	∼100/20	

[a] *Plaque appearance and diameter were observed against all sensitive hosts and the same results were obtained.*

PT-LP represented phage tail like particle. The phage images were taken with TEM at magnification of × 29,000. The black bar represented 100 nm.

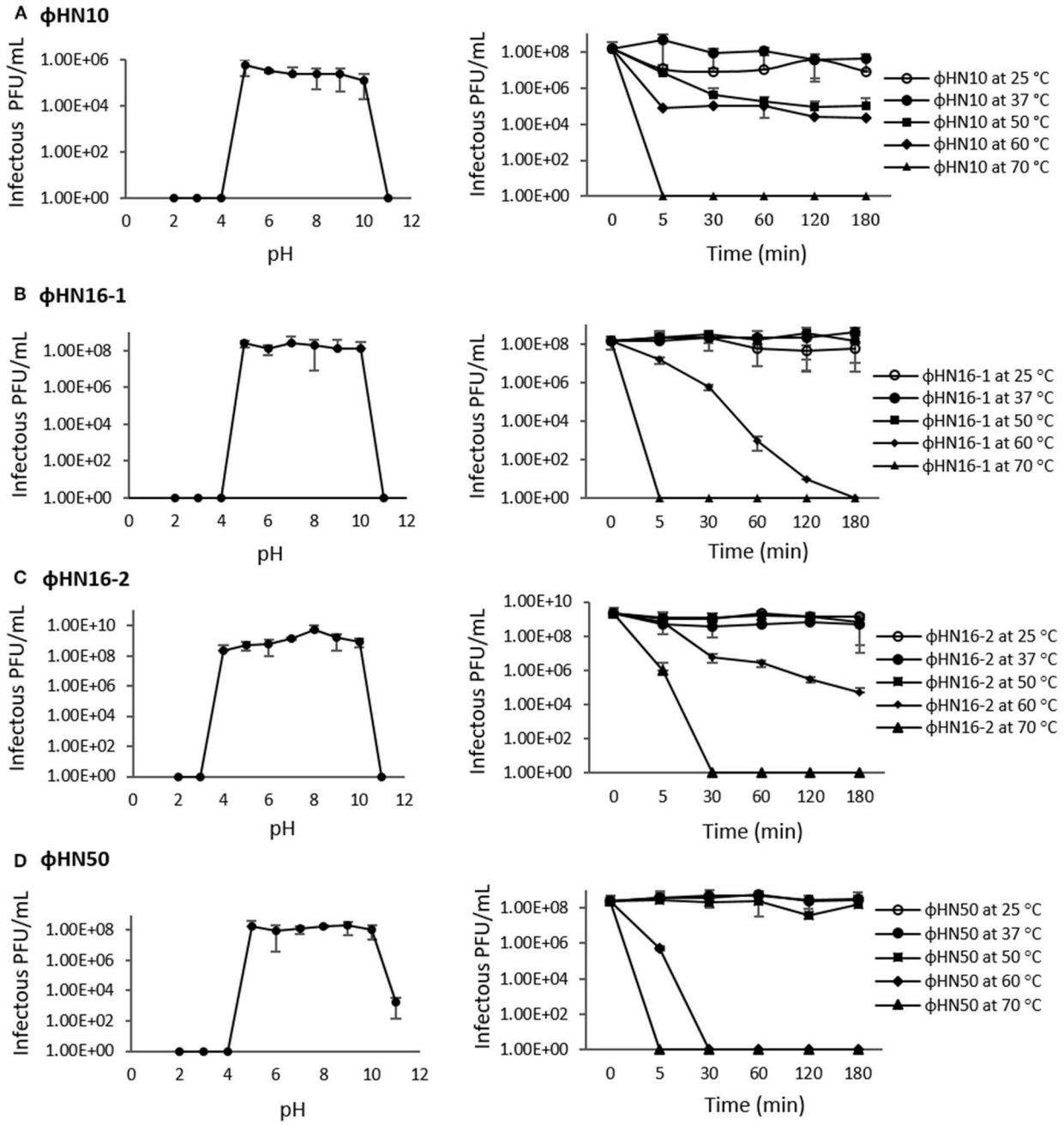


FIGURE 1 | pH and thermal stability test of phages. The first panel represented phage infectivity after incubation at different pH ranging from pH 2 to pH 11 at 37°C for 180 min. The second panel represented phage infectivity after treated with different temperature as indicated time point. Phage titer were enumerated by spot-titer assay. **(A–D)** represent phages ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50, respectively.

(**Figure 5**). This result indicated that the S-layer protein is likely to be the molecular target on bacterial surface for phage attachment.

DISCUSSION

The emergence of *C. difficile* hypervirulent strains (DePestel and Aronoff, 2013) and the limiting treatment options for CDI (Venugopal and Johnson, 2012) poses an urgent threat to public health. The development of alternative anti-infection modalities has become one of the highest priorities of clinical research. Phage therapy is one of potentials treatment for CDI due to its safety and specificity toward host bacteria. However, as has been known, no strictly virulent phage that infect *C. difficile* has yet been discovered. Therefore, it is necessary to identify and characterize a range of new phages,

which will provide a strong foundation of knowledge and a library of phage for further development and improvement of phages for therapeutic purposes. Here, we report the isolation and characterization of phages that target clinical strains of *C. difficile*. Although many investigators have focused on the extensive screening of *C. difficile* phages from environmental and clinical samples, there is no report on non-lysogenic phage thus far (Hargreaves and Clokie, 2014). Therefore, our work focused on inducible lysogenic *C. difficile* phages from clinical isolates. In order to reduce the massive use of inducing agents and laborious efforts from blind induction, we screened for the phage marker genes in all clinical strains in our collection using PCR. Two sets of degenerate primers targeting *C. difficile* myovirus and siphovirus *holin* genes were exploited as markers to track prophage on host genome. Of interest, prophage DNAs were detected at a very high frequency (91.8%) among clinical *C. difficile* isolates. The fact that high percentage of clinical *C. difficile* isolates harbored prophages might suggest intricate evolutionary relationship between phages with lysogenic life cycle and *C. difficile*. In fact, acquisition of many toxin genes in various toxin-producing bacteria, for example, *Staphylococcus aureus*, *Streptococcus pyogenes*, shiga toxin-producing *E. coli*, and *C. botulinum*, involves temperate phages (Fortier and Sekulovic, 2013).

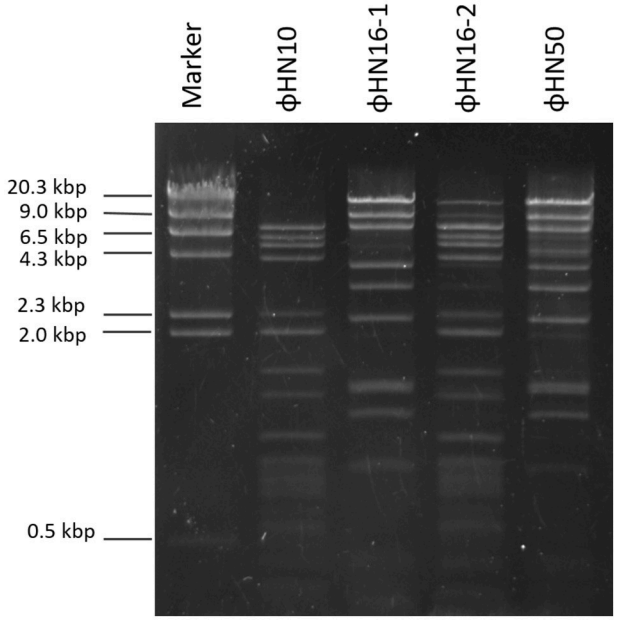


FIGURE 2 | Genomic DNA from phages were digested with the *Hind*III enzyme and analyzed on 1% agarose gel. The stained gel showed separated fragment of ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50. Lambda DNA digested with *Hind*III enzyme was included as a marker.

Our results indicated that not all prophages could be induced to produce free phage particles, even though the phage specific gene was detected in their genomes. It is the evident from PCR that not only complete bacteriophage genomes but also prophage-related fragments or defective phages could be detected. Others possible explanations may be due to the lack of the optimal inducing agent and induction conditions. However, the protocol for temperate phage induction with mitomycin C is commonly used in gram-positive bacteria (Goh et al., 2005; Fortier and Moineau, 2007; Shan et al., 2012). In this study, 5 temperate phages including ΦHN10, ΦHN16-1, ΦHN16-2, ΦHN50, and ΦHR24 were successfully induced from 4 lysogenic strains with mitomycin C. The lysogenic strain HN16 harbored 2 different temperate phages, with distinguishable morphology as well as unique restriction patterns (**Table 2** and **Figure 2**). All phages possessed a long, non-flexible, and contractile sheath tail, suggesting that they

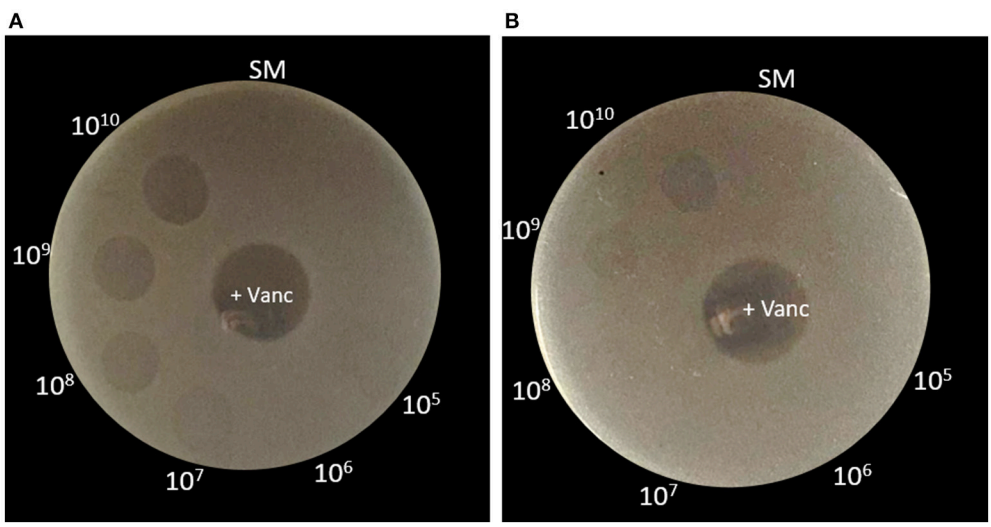


FIGURE 3 | Spot-titer assay showed difference in phage susceptibility between normal and defective bacterial hosts. **(A)** For normal HN2 cells, they were sensitive to phage at 10^5 PFU/ml. **(B)** Defective HN2 cells were sensitive to phage at 10^9 PFU/ml. +Vanc, Vancomycin as positive control; SM, buffer as negative control.

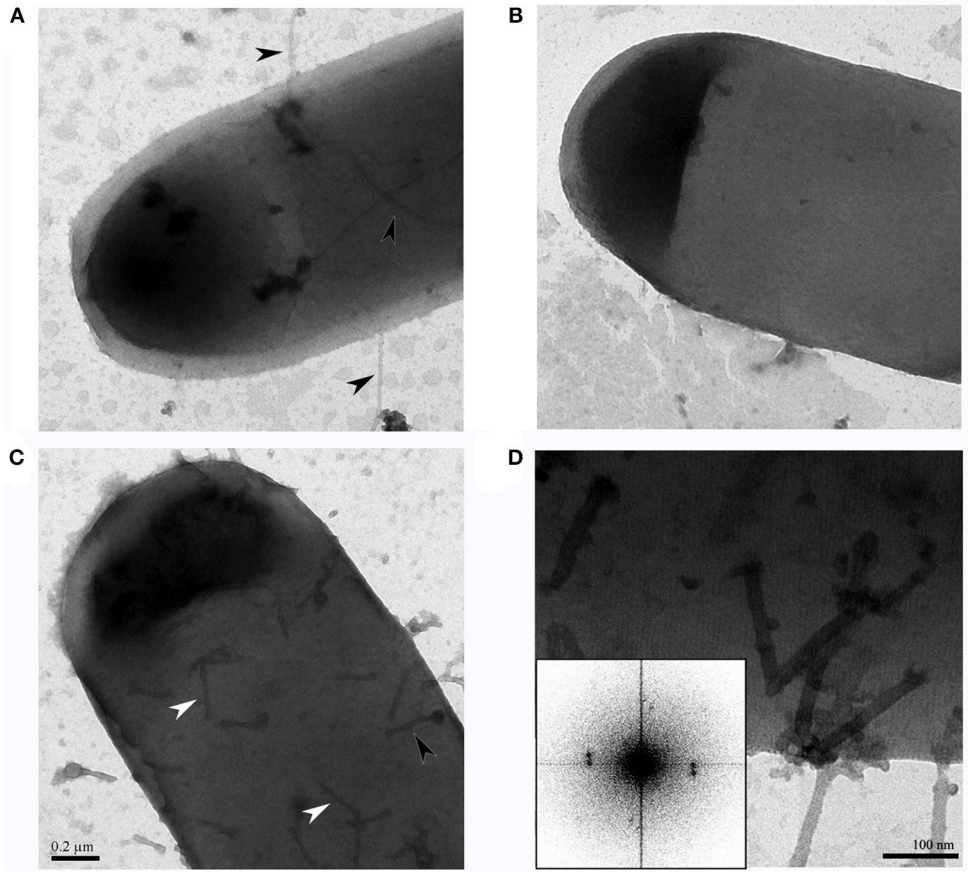


FIGURE 4 | TEM images revealed the binding site of *C. difficile* phages on the cell, **(A)** Intact *C. difficile* HN2 isolate contained pili as indicated by black arrowheads, **(B)** After mechanical disruption, *C. difficile* HN2 isolate showed absence of pili, **(C)** Intact and ghost particles of ΦHN10 phages attach on mechanical disrupted pili-less *C. difficile* host as depicted by black and white arrowheads respectively, scale bar for **(A–C)** is 200 nm **(D)** Expanded view of phage attached pili-less *C. difficile* revealed that phages adhered the to a mesh-like structure on the cell surface. Inset: Fourier transform pattern of the phage attached pili-less cell confirmed the existence of well-ordered S-layer of *C. difficile* with tetragonal lattice (Cerquetti et al., 2000), scale bar in **(D)** represented 100 nm.

belonged to Myoviridae family of the order Caudovirales, according to ICTV classification (Mayo and Horzinek, 1998). The phages size in this study were within a size range of previously reported of *C. difficile* myovirus (Goh et al., 2005; Fortier and Moineau, 2007; Meessen-Pinard et al., 2012; Nale et al., 2012; Shan et al., 2012). Moreover, as observed by several studies of *C. difficile* phage induction, the TEM analysis also revealed the PT-LPs with T-like shape. The T-like shape resembles a contractile tail of myophage (Fortier and Moineau, 2007; Nale et al., 2012). The existence of PT-LP was also reported in other bacterial species including *Vibrio* sp., and R-type pyocin of *Pseudomonas aeruginosa* (Lee et al., 1999; Gnezda-Meijer et al., 2005). The T-like shape particle has been demonstrated to possess bactericidal activity against other bacteria of the same species and provides competitive advantage to their hosts including in *C. difficile*, *P. aeruginosa*, *Budvicia aquatica,* and *Pragia fontium* (Šmarda and Benada, 2005; Sangster et al., 2015). Since, the PT-LPs lack the full phage genomes but still have capability to kill the specific target bacteria, they could also be a promisingly safe antimicrobial agent against CDI because there will be no concern regarding horizontal gene transfer between bacteria.

Four temperate phages including ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50 exhibited a narrow host range with the maximum number of infected host of only 6/92 isolates (6.5%) while ΦHN24 did not have any susceptible host. The narrow host range of *C. difficile* phage has also been described previously (Fortier and Moineau, 2007; Nale et al., 2012; Shan et al., 2012). Due to the lack of suitable propagating host, phages could not be replicated, posing as a major limitation for *C. difficile* phage study. There are two possible hypotheses that could explain the narrow host range of *C. difficile* phages. First, the host defense mechanisms that prevent phage infection including abortive system, restriction system, CRISPR/Cas system, and modification of phage-specific host receptor (Labrie et al., 2010; Bhaya et al., 2011). Second, the superinfection exclusion system, provided by existing prophage in the host genome, that inhibits secondary infection and infection of related phages. The narrow host

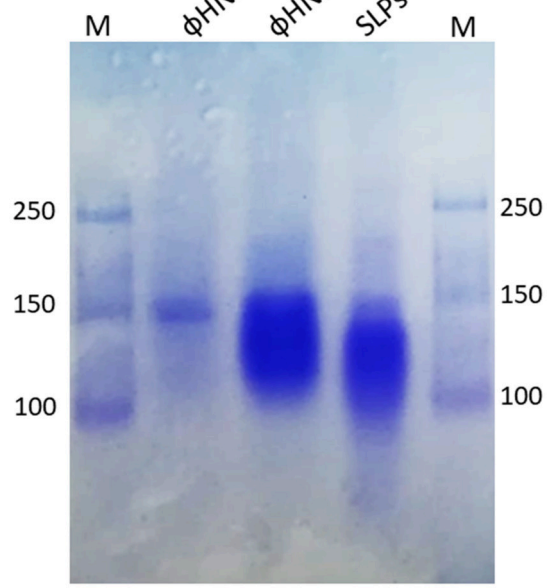


FIGURE 5 | Detection of S-layer proteins (SLPs) extracted from *C. difficile* strain HN2. Native-PAGE stained with Coomassie blue; M: standard protein marker, lane labeled ΦHN10 control prepared from phage ΦHN10 alone, lane labeled ΦHN10: SLPs was SLPs after preincubation with phage for 6 h, lane labeled SLPs control was SLPs prepared from *C. difficile* strain HN2 alone.

range would limit its application in CDI therapy. However, four temperate phages were induced from different isolates of the ribotype 017 lysogens and this particular ribotype was sensitive to all phages as well. Moreover, the ribotype 017 is the one of the highly prevalent and virulent ribotypes worldwide beside the ribotypes 027, 078, and 014 (Cairns et al., 2012). Therefore, the identification of phage against to this ribotype will certainly impact the prevention, and the understanding of phage-host interaction of this virulent ribotype.

Stability of phages should be concerned for development of phage as therapeutic treatment. Previous studies reported that factors such as temperature and pH play important roles on the survival of phages (Pirisi, 2000; Jepson and March, 2004; Silva et al., 2014). The pH influenced attachment, infectivity, intracellular replication and multiplication of phages (Pirisi, 2000; Jepson and March, 2004). Although the optimal pH condition for long terms storage of phage is neutral pH, all of isolated phages in this study have highest infection ability in various pH values ranging from 5 to 10. From this result, efficiency of phages should not be affected by either the storage condition or the pH of human body. Temperature is also one of the important factors that destroy the infectivity of phages. The results revealed that phages show maximum stability in the temperature range of 25–37°C. Most phages, including ΦHN16-1, ΦHN16-2, and ΦHN50, are still stable up to 50°C. Similar to pH, temperature can influence the efficiency of phages and it should be chosen carefully for storage and utilization condition.

The fact that restriction endonuclease are able to cleave the genomic DNA isolated from the phage suggesting that all four temperate phages including ΦHN10, ΦHN16-1, ΦHN16-2, and ΦHN50 have dsDNA genome, which is the major genetic material type reported in the order of *Caudovirales* (tailed phage; Horgan et al., 2010; Sekulovic et al., 2014; Nale et al., 2016). The distinct restriction pattern suggest that they are all different phage particles.

Phage requires binding of specific receptor on bacterial host for initiating the infection. The phage receptor has been shown to locate at different parts of bacterial host such as the walls of both of gram-positive (Xia et al., 2011) and gram-negative (Marti et al., 2013), appendages including pili and flagella (Shin et al., 2012; Marti et al., 2013). However, the specific receptors for *C. difficile* phage have never been reported so far. Here, we focused on pili and the other cell wall appendages on the cell surface of *C. difficile* host. The mechanical shearing method was applied for preliminary identification of phage receptor on bacteria host. Typically, mechanical disruption is used for pili detachment from *E. coli* (Korhonen et al., 1980) and removing of thick cell wall of gram-positive bacteria such as *Bacillus subtilis* (Vandeventer et al., 2011). Mechanical shearing of HN2 bacterial host resulted in the decline in infectivity of ΦHN10 when compared with the normal cell host determined by spot-titer assay (**Figure 3**). Moreover, TEM analysis revealed that ΦHN10 attachment at mesh-like structure of bacterial cell wall, not at the pili (**Figures 4C,D**). Our Fourier calculation confirmed that the mesh-like structure on the cell wall surface of *C. difficile* is S-layer, containing various proteins or glycoproteins (Fagan and Fairweather, 2014). Although the results suggested that the S-layer on bacterial cell wall might be potentially a phage receptor, wall-teichoic acid (WTA) and lipoteichoic acid (LTA) could also the possible candidate receptor among the Gram-positive bacteria (Bertozzi Silva et al., 2016). Therefore, S-layer proteins were prepared and used in the phage receptor study. In this study we used the phage binding assay to investigate the cognate receptor of ΦHN10 on its host cell surface. This assay suggested that ΦHN10 can specifically bind to S-layer proteins, indicating direct interaction between the S-layer proteins to ΦHN10 phage. To our knowledge, this was the first evidence suggesting S-layer as a phage receptor of *C. difficile*. This work not only offers insights into the diversity of phages targeting *C. difficile* clinical isolates but also provide detailed information on the molecular interaction between phage and *C. difficile*.

AUTHOR CONTRIBUTIONS

SC conceived and designed the study. WP, PO, TP, PW, and SC performed the experiments. TJ, PK, and SS helped with the experimental assays. WP, TP, and SC wrote the paper. PO and TJ edited the manuscript. SC supervised the project. All authors have read and approved the manuscript.

ACKNOWLEDGMENTS

This research project is supported by Governmental budget through Mahidol University. Further support was generously provided by the Science Achievement Scholarship of Thailand (SAST) and Sri Trang Thong Scholarship, by Faculty of Science, Mahidol University for WP and TP, respectively.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01701/full#supplementary-material

Supplementary Figure 1 | Prophage carriage *C. difficile* detection. Gel electrophoresis revealed representative PCR products of *holin* gene in *C. difficile* isolates. **(A,B)** shown *holin* gene of myovirus and siphovirus, respectively.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 September 2018
doi: 10.3389/fmicb.2018.02080

An Engineered Synthetic Biologic Protects Against *Clostridium difficile* Infection

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Morbidity and mortality attributed to *Clostridium difficile* infection (CDI) have increased over the past 20 years. Currently, antibiotics are the only US FDA-approved treatment for primary *C. difficile* infection, and these are, ironically, associated with disease relapse and the threat of burgeoning drug resistance. We previously showed that non-toxin virulence factors play key roles in CDI, and that colonization factors are critical for disease. Specifically, a *C. difficile* adhesin, Surface Layer Protein A (SlpA) is a major contributor to host cell attachment. In this work, we engineered Syn-LAB 2.0 and Syn-LAB 2.1, two synthetic biologic agents derived from lactic acid bacteria, to stably and constitutively express a host-cell binding fragment of the *C. difficile* adhesin SlpA on their cell-surface. Both agents harbor conditional suicide plasmids expressing a codon-optimized chimera of the lactic acid bacterium's cell-wall anchoring surface-protein domain, fused to the conserved, highly adherent, host-cell-binding domain of *C. difficile* SlpA. Both agents also incorporate engineered biocontrol, obviating the need for any antibiotic selection. Syn-LAB 2.0 and Syn-LAB 2.1 possess positive biophysical and *in vivo* properties compared with their parental antecedents in that they robustly and constitutively display the SlpA chimera on their cell surface, potentiate human intestinal epithelial barrier function *in vitro*, are safe, tolerable and palatable to Golden Syrian hamsters and neonatal piglets at high daily doses, and are detectable in animal feces within 24 h of dosing, confirming robust colonization. In combination, the engineered strains also delay (in fixed doses) or prevent (when continuously administered) death of infected hamsters upon challenge with high doses of virulent *C. difficile*. Finally, fixed-dose Syn-LAB ameliorates diarrhea in a non-lethal model of neonatal piglet enteritis. Taken together, our findings suggest that the two synthetic biologics may be effectively employed as non-antibiotic interventions for CDI.

Keywords: ***Clostridium difficile,*** **infectious diarrhea, synthetic biology, surface layer protein,** ***Lactobacillus***

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *22 May 2018*
Accepted: *14 August 2018*
Published: *05 September 2018*

Citation:
Vedantam G, Kochanowsky J, Lindsey J, Mallozzi M, Roxas JL, Adamson C, Anwar F, Clark A, Claus-Walker R, Mansoor A, McQuade R, Monasky R, Ramamurthy S, Roxas B and Viswanathan VK (2018) An Engineered Synthetic Biologic Protects Against Clostridium difficile Infection. Front. Microbiol. 9:2080. doi: 10.3389/fmicb.2018.02080

INTRODUCTION

The fastidiously anaerobic, Gram-positive bacillus *Clostridium difficile* is currently the largest contributor to healthcare-associated infections in US hospitals (McDonald et al., 2018). In the United States, an estimated 450,000 cases of *C. difficile* infections (CDI) occur annually (Lessa et al., 2015), costing the healthcare system ≥ $5.4 billion (Desai et al., 2016). CDI symptoms range from mild/moderate diarrhea, which can progress to serious sequelae including pseudomembranous colitis. Antibiotic suppression of gut flora facilitates colonization by *C. difficile* spores that are commonly present in the environment, which germinate into vegetative cells that produce damaging toxins during late growth phase. Toxigenic (toxin-producing) *C. difficile* strains harbor a 19.6 kb genomic island called the Pathogenicity Locus (PaLoc), that encodes the glucosyltransferase toxins TcdA (309 kDa) and TcdB (267 kDa), which target host-cell G-proteins (Lyras et al., 2009). A third ADP ribosylase toxin CDT (Binary toxin) is present in some strains.

Clostridium difficile epidemiology has altered markedly in the past 20 years. Highly virulent strains, associated with severe disease, increased recurrence rate(s) and community onset, have become more prevalent (Ofori et al., 2018). Common human outbreak-associated strains are typed as North American Pulsed-Field type 1 (NAP1) and PCR ribotype 027 (NAP1/027) (Loo et al., 2005). Common veterinary strains (now also isolated from humans) belong to the NAP7/NAP8 clade (Ribotype 078) (Moono et al., 2016). There have been several worldwide outbreaks of NAP1/027/BI CDI since 2002 (Loo et al., 2005).

Bacterial adherence is an important *C. difficile* virulence attribute, with Surface-Layer proteins (SLPs; also known as cell-wall proteins, CWPs) playing key roles. *C. difficile* elaborates up to 29 different SLPs, which are displayed in para-crystalline architecture on the cell surface. *C. difficile* SLPs are also implicated in immune modulation; thus, they are critical non-toxin virulence factors (Bianco et al., 2011; Bruxelle et al., 2016). While SLPs have been proposed as anti-CDI vaccine candidates, many groups (including ours) have reported variability in SLP epitope antigenicity (Biazzo et al., 2013).

Surface-layer protein A (SlpA) and its orthologs are abundant members of the CWP complex in clostridia and lactobacilli. We previously published that pre-incubating human intestinal epithelial cells with *C. difficile* SlpA-enriched preparations, or purified SlpA, or bacteria with anti-SlpA antisera, reduced > 50% *C. difficile* adherence in a dose-dependent manner, implicating SlpA as a major adhesin (Merrigan et al., 2013). Notably, SlpA from a non-toxigenic *C. difficile* isolate blocked adhesion of the strain from which it was derived, as well as a phylogenetically unrelated, non-cognate strain. The degree of adherence inhibition was similar irrespective of the challenge isolate. SlpA is a heterodimer of high-and low-molecular weight (HMW and LMW) subunits. Both subunits bind independently to intestinal cells, with the LMW subunit displaying higher binding efficiency. This guided the engineering of our synthetic strains.

The typical requirement of antibiotics to precipitate CDI, as well as the remarkable efficacy of fecal microbiota transplants in treating refractory CDI, point to one unequivocal conclusion: colonization resistance is an effective and 'natural' method to combat CDI (Austin et al., 2014; Terveer et al., 2018). At a practical level, however, fecal transplantation may not be the ideal therapeutic option for all CDI patients (Wang et al., 2016; Gardiner et al., 2018). Alternate approaches that exploit colonization resistance for CDI prevention and/or cure are active areas of investigation (Allen-Vercoe and Petrof, 2013; Petrof and Khoruts, 2014). Probiotics, particularly lactic acid bacteria (LAB), have been considered as safe, palatable options to confer colonization resistance. LAB occupy the same gut niches as *C. difficile* in humans and rodents (Marco et al., 2007), and proliferate to the same or greater extent as *C. difficile* (Chiu et al., 2006). Although LAB can reduce symptoms in some patients, meta-analyses and large-cohort studies suggest variability in LAB protection against CDI (Mergenhagen et al., 2014; Goldenberg et al., 2017). The basis for this variability is unknown, and may reflect inconsistent gut colonization and persistence. Since LAB harbor SLP orthologs and can express heterologous SLP molecules on their surface (Raha et al., 2005; Zhu et al., 2010), we exploited these properties to engineer targeted synthetic biologics with enhanced colonization and immune elicitation properties.

MATERIALS AND METHODS

Cell Lines

The human intestinal epithelial cell line $C2_{BBe}$, a brush border-expressing Caco-2 sub-clone (Peterson and Mooseker, 1992), was used in this study and cultured as previously reported (Roxas et al., 2014).

Bacterial Strains and Plasmids

All strains and plasmid are described in **Table 1**. LAB were purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). Specifically, *Lactobacillus casei* strain 334 (Orla-Jensen; Dellaglio et al., 2002), and *Lactobacillus acidophilus* strain 4356 (Roussel et al., 1993) were used for these studies. LAB were grown in De Man, Rogosa and Sharpe (MRS) broth (Duong et al., 2011) and incubated at 30°C in the presence of 5% CO_2. Bacteria were cultured for 3–5 days to reach saturation [$\geq 1.0 \times 10^8$ colony forming units (CFU) per mL].

Lactobacillus acidophilus and *L. casei* strains ferment the dextrose in MRS to distinct products, and the corresponding pH changes can be detected by including bromophenol blue into the media (MRS-BPB) (Lee and Lee, 2008). *L. acidophilus*, a homo-fermenter, metabolizes dextrose to lactic acid, and the plates remain violet/blue (pH > 4.6); *L. casei*, a hetero-fermenter converts dextrose to acetic acid, and the drop in pH (<3.0) results in a color change to yellow/white. Further, *L. casei*, unlike *L. acidophilus*, can ferment mannitol, and this can be verified by growth on Purple Broth Base (Difco™ Becton, Dickinson and Company Sparks, Glencoe, MD, United States); the acidic change resulting from mannitol fermentation causes the pH indicator bromocresol purple to turn yellow.

The *slpA C. difficile*/*L. acidophilus* "chimera" fragment (**Figure 1A**) was designed with a strong lactic-acid-bacterial

(LAB) promoter [endogenous to the phosphoglycerate mutase (*pgm*) gene in plasmid pTRK848 (Duong et al., 2011)], a lactic-acid-bacterial Shine-Dalgarno (ribosome binding site) sequence (Duong et al., 2011), a signal sequence from a *Lactobacillus acidophilus* S-layer protein, a codon-optimized *C. difficile* strain 630 host-cell-binding fragment, and the *L. acidophilus* SlpA-ortholog cell-wall-binding domain(Michon et al., 2016). The entire fragment (F1) was chemically synthesized (DNA 2.0, now ATUM, Newark, CA, United States), and cloned into the DNA 2.0 maintenance vector pJ241 to yield pMGM13. A second DNA fragment (F2) comprising a broad host-range temperature-sensitive origin of replication (*repA*), and a chloramphenicol resistance gene (*catP*) was also chemically synthesized based on sequence information obtained from the broad host-range plasmid [pKS1, Steen et al., 2010]. This fragment was self-ligated to form pMGM11 ("empty vector"). The two synthesized fragments F1 and F2 harbored *Pme*I and *Fse*I restriction sites at their termini; following digestion with those enzymes, linear F1 and F2 were ligated using T4 DNA ligase (Sigma, St. Louis, MO, United States) to generate pMGM14. Plasmid integrity

TABLE 1 | Bacterial strains and plasmids used in this study.

Bacteria	Aliases	Species	Genotype	Resistance	Source/Notes
ATCC 393	12473, Orland L-323	*Lactobacillus casei*	Wild type		ATCC
ATCC 4356	Hansen	*Lactobacillus acidophilus*	Wild type		ATCC
DH10B		*Escherichia coli*	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80lacZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara, leu*)7697 *galU galK* λ- *rpsL nupG* /pMON14272/pMON7124		DNA 2.0 (ATUM) Maintenance strain
GC5		*Escherichia coli*	*recA1 endA1 tonA1*		Genesee Scientific
GV1095		*Escherichia coli*	GC5 + pMGM14	Chloramphenicol	This study
GV1096		*Escherichia coli*	GC5 + pMGM12	Chloramphenicol	This study
GV1097		*Escherichia coli*	DH10B + pMGM13	Kanamycin	This study
GV1099		*Lactobacillus casei*	ATCC393 + pTRK848	Erythromycin	This study
GV1100		*Lactobacillus casei*	ATCC393 + pMGM14	Chloramphenicol	This study
GV1101		*Lactobacillus acidophilus*	ATCC4356 + pMGM12	Chloramphenicol	This study
GV1102 (group)		*Lactobacillus acidophilus*	ATCC4356 + pMGM14	Chloramphenicol	This study
Top 10		*Escherichia coli*	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80lacZΔM15 Δ *lacX74 recA1 araD139* Δ*(ara leu)*7697 *galU galK rpsL (StrR) endA1 nupG*	Streptomycin	Cloning strain, Thermo Fisher Scientific
6396	Ribotype 012; strain 630	*C. difficile*	Wild type		Gerding Lab, Edward Hines Jr. VA Hospital, Illinois
1470	Ribotype 017	*C. difficile*	Wild type		ATCC
R10079	Ribotype 020	*C. difficile*	Wild type		Cardiff-ECDC*
R20291	Ribotype 027	*C. difficile*	Wild type		Cardiff-ECDC
R26222	Ribotype 078	*C. difficile*	Wild type		Cardiff-ECDC
Plasmids					
pTRK848			Expression vector based on a pWV01 origin of replication	Erythromycin	Kok et al., 1984; Duong et al., 2011
pKS1			Broad host-range plasmid pWV01 with a temperature-sensitive *repA* allele	Kanamycin and erythromycin	Shatalin and Neyfakh, 2005
pMGM12			pKS1 + *catP;* self-ligated	Chloramphenicol	This study
pMGM13			pJ241 – DNA 2.0 (now ATUM) maintenance vector harboring the *slpA* chimera fragment	Kanamycin	This study
pMGM14			pMGM12 harboring the *Pme*I-*Fse*I *slpA* chimera-containing fragment from pMGM13	Chloramphenicol	This study

**Cardiff-European Centre for Disease Prevention and Control [ECDC] C. difficile collection.*

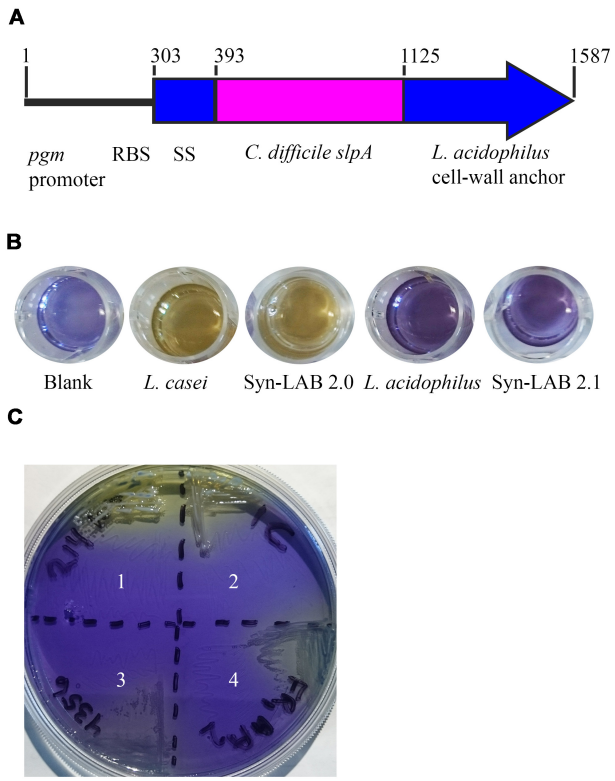


FIGURE 1 | Design and confirmation of Syn-LAB strains. **(A)** Schematic of the *slpA* chimera. Expression is driven by the *L. acidophilus* (LA) *pgm* promoter and ribosome-binding site. The chimera encodes the LA signal sequence (SS), the codon-optimized *C. difficile* extracellular and antigenic SlpA fragment, and the LA Slp cell wall anchor. Syn-LAB strain confirmation via differential growth on mannitol-MRS broth **(B)** and mMRS-BPB agar **(C)**. 1, *L. casei* parent strain; 2, Syn-LAB 2.0; 3, *L. acidophilus* parent strain; 4, Syn-LAB 2.1.

of pMGM14 was confirmed by complete DNA sequencing. All plasmids were transformed into *Escherichia coli* strains DH10B, TOP10 or NCK1753 for maintenance or propagation purposes, or into *E. coli* GC5 prior to extraction for electroporation. All plasmids were PCR-verified prior to any use in LAB.

Lactic Acid Bacterial Transformation

The pMGM14 plasmid described above was first extracted from the *E. coli* storage strain, and 10 μg used for each LAB transformation. *Lactobacillus acidophilus* strain 4356 and *Lactobacillus casei* 343 were propagated in Mann-Rogosa-Sharpe (MRS) medium (Remel, Lenexa, KS, United States) per the method of Walker et al. (1996) and Kim et al. (2005). In brief, LAB strains were grown to saturation at 37°C in 5% CO_2, sub-cultured at a 1:50 dilution, and re-grown using the same process two more times. To shear cell-wall proteins, and prepare the resulting protoplasts for electroporation, a fourth sub-culture was grown for 15 h in MRS supplemented with 1% glycine, and then sub-cultured in the same medium at a 1:50 dilution for an additional 6 h. Bacteria were harvested using centrifugation, and chilled pellets subjected to electroporation with 10 μg plasmid DNA, followed by recovery in MRS broth for 3 h, and growth on MRS-agar + 5 μg/mL chloramphenicol (Sigma, St. Louis, MO, United States) at 30°C in 5% CO_2. Plasmid was extracted from one-half of a colony of multiple purified transformants, and verified via DNA sequence analysis. The remaining bacterial colonies were propagated in the presence of chloramphenicol at 42°C, the non-permissive temperature, at which *repA* is non-functional, thus selecting for integrants. For pMGM14-based transformants, putative integrants were purified, and assessed for chimeric *slpA* presence by PCR. All confirmations were performed at the non-permissive temperature. Three independently isolated and PCR-verified identical transformants were bio-banked for the *L. casei* strain (herein referred to as Syn-LAB 2.0 clones), and seventeen independently isolated and PCR-verified identical transformants for the *L. acidophilus* strain (herein referred to as Syn-LAB 2.1 clones). For *in vitro* studies only, all LAB strains were propagated at the non-permissive temperature with antibiotic selection as appropriate (5 μg/mL chloramphenicol). However, for all *in vivo* studies, while strains were propagated as above, no selection antibiotic was administered to the animals.

An identical procedure was used to generate empty-vector harboring LAB strains (**Table 1**); these strains were PCR-verified, and used only for *in vivo* studies. Plasmids were maintained episomally; the lack of homology with the LAB host limited the possibility of integration of vector sequences into the chromosome.

Transepithelial Electrical Resistance (TEER) Measurements

Polarized human intestinal epithelial cells ($C2_{BBe}$; Peterson and Mooseker, 1992) were grown on 0.33-cm^2 collagen-coated Corning™ Transwells (Thermo Fisher Scientific) for 14 days. Monolayers were treated apically with 1.42×10^7 CFU/well and 1.96×10^7 CFU/well of parental and Syn-LAB 2.0 respectively. Measurements were made every hour for 7 h, and at 24 h post-treatment using an epithelial volt-Ohm voltmeter (World Precision Instruments, Sarasota, FL, United States), and TEER calculated by applying Ohm's Law. An identical setup was used when testing Syn-LAB 2.1 and its *L. acidophilus* parent strain.

Host Cell Survival Measurement

$C2_{BBe}$ monolayers were treated with media alone, the parent LAB strain, or the corresponding Slp chimera-expressing Syn-LAB derivatives, and host cell viability was assessed using the propidium iodide (PI) uptake assay as described previously (Roxas et al., 2012). Briefly, PI (2 μg/ml; Molecular Probes) was added to the treated cells, and fluorescence measured after 30 min using a microplate reader (Synergy HT; BioTek instruments, Winooski, VT, United States). To estimate maximal PI uptake, a set of wells were treated with 70% methanol prior to PI treatment.

Immunoblot Analyses

Surface layer proteins (Slp) were extracted from *Lactobacillus* parent and Syn-LAB strain saturated cultures (OD_{600nm} = 1.5) using 0.2M glycine (pH2.2), as described by Calabi et al. (2001).

For dot blot analyses, 31.25, 62.5, 125, 500 ng, 1 μg, and 2 μg of total protein were blotted on nitrocellulose membranes (Bio-Rad, Richmond, CA, United States). Slp extracts (5–10 μg) were also separated on 4–20% TGX^{TM} pre-cast protein gels (Bio-Rad, Richmond, CA, United States). Separated proteins were transferred to 0.2-μm nitrocellulose membranes (Transblot Cell Apparatus, Bio-Rad). Blots were blocked with 5% non-fat milk in Tris-buffered saline containing Tween 20 (TBST) for 1 h, incubated with antiserum specific to *C. difficile* SlpA (raised against the *C. difficile* strain 630 SlpA (Merrigan et al., 2013) overnight at 4°C and in horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at room temperature (Sigma–Aldrich, St. Louis, MO, United States). Membranes were washed five times for 5 min in blocking solution between each incubation step and developed with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, United States).

Immunofluorescence Microscopy

SlpA chimera expression in *L. casei* WT and Syn-LAB 2.0 and Syn-LAB 2.1 strains was evaluated via immunofluorescence staining using antiserum specific to *C. difficile* SlpA. *Lactobacillus sp* cultures were allowed to settle for 10 min in 12-well plates with poly-L-lysine-coated coverslips. Unattached bacteria were removed, and samples were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, quenched with 50 mM NH_4Cl and 0.125M glycine in PBS for 15 min, and blocked with 5% IgG-free bovine serum albumin (BSA) in PBS for 1 h. Samples were incubated with antiserum specific to *C. difficile* SlpA overnight at 4°C, and then washed three times with 1% IgG-free BSA in PBS. Secondary antibodies (Alexa 488-conjugated goat anti-rabbit IgG antisera; Thermo Fisher Scientific, Waltham, MA, United States) were added at 8 μg/ml in 5% IgG-free BSA for 1 h. Samples were mounted in ProLong Diamond Antifade reagent (Thermo Fisher Scientific, Waltham, MA, United States). Intestinal tissue samples (ileum, cecum, and colon) from LVG Golden Syrian Hamsters (Charles River Laboratories, San Diego, CA, United States) treated with Syn-LAB 2.0 were frozen in OCT embedding medium (Tissue-Tek, Sakura Finetek, Torrance, CA, United States) and stored at −80°C. OCT-mounted tissue samples cut at 3 micron thickness were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min, and processed for SlpA immunofluorescence staining as described above. Samples were stained with 4,6-diamidino-2-phenylindole (DAPI) prior to mounting in ProLong Diamond Antifade reagent. Images were captured using EVOS® FL Imaging System (Thermo Fisher Scientific, Waltham, MA, United States) or DeltaVision Elite Deconvolution Microscope (GE Healthcare, Pittsburgh, PA, United States).

Flow Cytometry

Parent and transformed *Lactobacillus* sp strains were cultured in MRS broth as described above, and subjected to Gram's staining to verify purity and morphology. Bacteria were pelleted by centrifugation at 4000 *g* for 2 min. Bacterial pellets were washed gently three times with blocking solution (2% IgG-free BSA in PBS) and then incubated with antiserum specific to *C. difficile* SlpA for 30 min. Secondary antibodies (Alexa Fluor 555-conjugated goat anti-rabbit IgG antisera; Thermo Fisher Scientific, Waltham, MA, United States) were added at 8 μg/ml in 2% IgG-free BSA for 30 min. Samples were washed three times with blocking solution after each antibody incubation step. Stained samples were re-suspended in blocking solution at 10^6cells/mL density and analyzed via flow cytometry using a BD FACSCANTO II machine (BD Biosciences, San Jose, CA, United States). List mode data files consisting of 10,000 events gated on FSC (forward scatter) vs. SSC (side scatter) were acquired and analyzed using FACSDiva 8.0.1 software (BD Biosciences, San Jose, CA, United States). Appropriate electronic compensation was adjusted by acquiring the cell populations stained with the fluorophore, as well as an unstained control.

Golden Syrian Hamster Studies

All hamster studies were approved by the Institutional Animal Care and Use Committee of the University of Arizona. The Golden Syrian hamster model was employed to study both colonization/shedding and protection conferred by Syn-LAB strains. For all studies, male hamsters (6–8 weeks; 90–110 g weight) were used.

Shedding studies: Prior to any treatment, hamster stool plated on MRS yielded no colonies, confirming that the animals were devoid of endogenous *Lactobacillus* bacteria. Animals received a daily dose of 10^8 Syn-LAB 2.0 or 10^8 Syn-LAB 2.1 respectively. Feeding and enumeration were continued for 6 days. For Syn-LAB 2.0-treated animals only, oral clindamycin (prescription solution; clindamycin sulfate; University of Arizona Pharmacy; 30 mg/kg) was administered on Day 4, and LAB detection monitored until Day 6. Fecal pellets were collected daily, and pellets were re-suspended in PBS, homogenized, serially diluted and plated on the appropriate Syn-LAB selective medium containing chloramphenicol. Colonies were detected only in stool samples from Syn-LAB-treated animals, and not from untreated controls. Shedding from all animals was statistically indistinguishable. For added confirmation, select colonies were 16S PCR-verified for *L. casei* as well as the presence of the chimeric *slpA*.

Challenge studies: These were performed in two modalities, a "fixed-dose" and a "continuous dose" format. All animals received clindamycin (prescription solution; clindamycin sulfate; University of Arizona Pharmacy; 30 mg/kg). 1000 spores of *C. difficile* strain CD630 was used in the challenge studies where indicated, and 10^8 CFU LAB was used wherever indicated. Group 1 animals received clindamycin on day -3 but no other intervention (black line in **Figure 8**). Group 2 hamsters received clindamycin (day -3) and *C. difficile* challenge (day 0), but no LAB treatment (blue line in **Figure 8**). Group 3 hamsters received *L. casei* parent strain/empty vector on days -6, -5, -4, -2, -1, and 0, and clindamycin on day -3, followed by *C. difficile* challenge on day 0 (magenta line in **Figure 8**). Group 4 hamsters received Syn-LAB 2.0 on days -6, -5, -4, -2, -1, and 0, clindamycin on day -3, and *C. difficile* challenge on day 0 (green line in **Figure 8**).

For continuous-dose studies, the clindamycin dose and timing was similar to that above, and only the "*C. difficile,*" "Empty Vector" and "LAB" groups as above were evaluated. Both "Empty

Vector" and Syn-LAB 2.0 ("LAB") were continuously dosed at 10^8 CFU per animal per day starting at Day -6 before infection, until death/euthanasia.

Where appropriate, infections commenced 72 h post-antibiotic administration, and the challenge strain used was *C. difficile* strain 630 (1000 spores; orally administered in PBS). Animals were monitored for disease symptoms (wet-tail, ruffled coat, lethargy) through the course of the studies. Moribund hamsters or those meeting the criteria for euthanasia were administered 270 mg/kg commercial euthanizing solution (Euthanasia III, MedPharma Inc, Pomona, CA, United States). Euthanized hamsters were dissected for visualization of gross pathology, and cecal contents harvested and plated on selective medium for recovery and molecular typing of *C. difficile* (using 16s-23s rDNA intergenic fragment profiling and comparison with the organisms used for infection). In all studies, and all groups, fecal pellets were also collected daily, re-suspended in PBS, homogenized, serially diluted and plated on *C. difficile* or *L. casei* selective medium as appropriate.

Immune response studies: for these experiments, age- and weight-matched Golden Syrian hamsters (at least 3 per group) were administered 10^8 CFU Syn-LAB 2.0 daily, or left untreated, for 21 days. Animals were then euthanized, whole blood harvested via cardiac puncture, and serum immediately retrieved after blood was centrifuged at 1000 *g* for 10 min. This material was aliquoted and stored at −80°C until further use. For immune response assessments, the same methodology as immunoblotting above was used, but serum from Syn-LAB or mock-treated animals was used as the source of primary antibody.

Neonatal Piglet Studies

All piglet studies were approved by the Institutional Animal Care and Use Committee of the University of Arizona; we assessed Syn-LAB 2.1 safety, tolerability and efficacy in protecting against *C. difficile* challenge. Newborn male and female piglets were obtained via assisted delivery from a local antibiotic-free, small-volume farm, and transferred to the University of Arizona Central Animal Facility within 2 h of birth. On Day 2 post-birth, piglets were treated with oral vancomycin (50 mg/kg; prescription solution, University of Arizona Pharmacy) to ablate any pre-existing *C. difficile* colonization. On day 6 post-birth, piglets were administered 10^{10} Syn-LAB 2.1 in milk replacer every 8 h. On day 7, a subset of animals was administered a non-lethal dose of 1000 *C. difficile* spores of strain 630. Monitoring included checks every 8 h thereafter, with weight, stimulus response and dehydrations scores recorded. Upon completion of the study, piglets were anesthetized with Ketamine/Xylazine, and then humanely euthanized with commercial euthanizing solution (Euthanasia III, MedPharma Inc, Pomona, CA, United States) followed by cardiac puncture. Histologic analyses included standard hematoxylin-eosin staining of colonic tissues following standard methodologies (Kiernan, 2008), and immunofluorescence staining of tissues with anti-*C. difficile* SlpA serum as described in detail above for visualization of Syn-LAB 2.0.

Statistical Analysis

Multiple statistical tests were employed and utilized the Excel-Stat application to determine significance for experiments involving quantitation. For growth and bacterial burden, Student's *t*-tests were performed to compute differences between parental and Syn-LAB strains, and errors bars calculated from standard deviation(s). For *in vivo* studies, Kaplan–Meier survival curves were computed followed by Log-Rank tests for *post hoc* analyses.

RESULTS

Construction of *C. difficile*/Lactic Acid Bacterium (LAB) SlpA Chimera-Encoding Plasmids and Generation of Syn-LAB Strains

A 7740-base pair (bp) shuttle vector was assembled from chemically synthesized fragments. The vector has the following features: a temperature-sensitive *repA* allele and a chimeric *slpA* (**Figure 1A**) that includes; (1) a strong *L. acidophilus* (LA) promoter [upstream sequences of the phosphoglycerate mutase (*pgm*) gene (base pairs 178339-178600 of the *L. acidophilus* NCFM genome, Genbank accession number CP000033.3 (Altermann et al., 2005; Duong et al., 2011) (2) an LA Shine-Dalgarno (ribosome binding site) sequence (CCTGCA); and (3) sequences encoding a chimeric SlpA that includes a LA signal sequence (amino acids 1–30 of LA SlpA) (Altermann et al., 2005), codon-optimized *C. difficile* strain 630 LMW SlpA host-cell-binding region (amino acids 1–243) (Karjalainen et al., 2002), and the LA cell-wall-binding domain (amino acids 291–444) (Altermann et al., 2005). Precise engineering of the entire 7740 base pair vector was confirmed by complete DNA sequencing.

Lactic acid bacterial strains were individually transformed with this shuttle vector. All transformants were recovered at the permissive temperature (30°C) where RepA is functional, confirmed by biochemical tests and selective plating (**Figures 1B,C**), and further confirmed by multiple PCR tests (not shown). Finally, transformants were propagated with selection at the non-permissive temperature (37–42°C) that allows for recovery of integrants. A total of 3 independently isolated *Lactobacillus casei* transformants were obtained (herein collectively referred to as Syn-LAB 2.0 clones). Seventeen independently isolated *Lactobacillus acidophilus* transformants were also obtained (herein referred to as Syn-LAB 2.1 clones). Syn-LAB clones of each LAB species were confirmed via PCR, phenotypic (growth; not shown) and biochemical tests (**Figures 1B,C**), and bio-banked. The unique carbohydrate fermentation profiles were exploited to readily distinguish between the two species: *L. casei* can ferment mannitol, and converts dextrose to acetic acid, and the corresponding media acidification manifested as a color change to yellow/white in the presence of appropriate pH indicators. *L. acidophilus,* on the other hand, does not ferment mannitol, and converts dextrose to lactic acid; the media remained

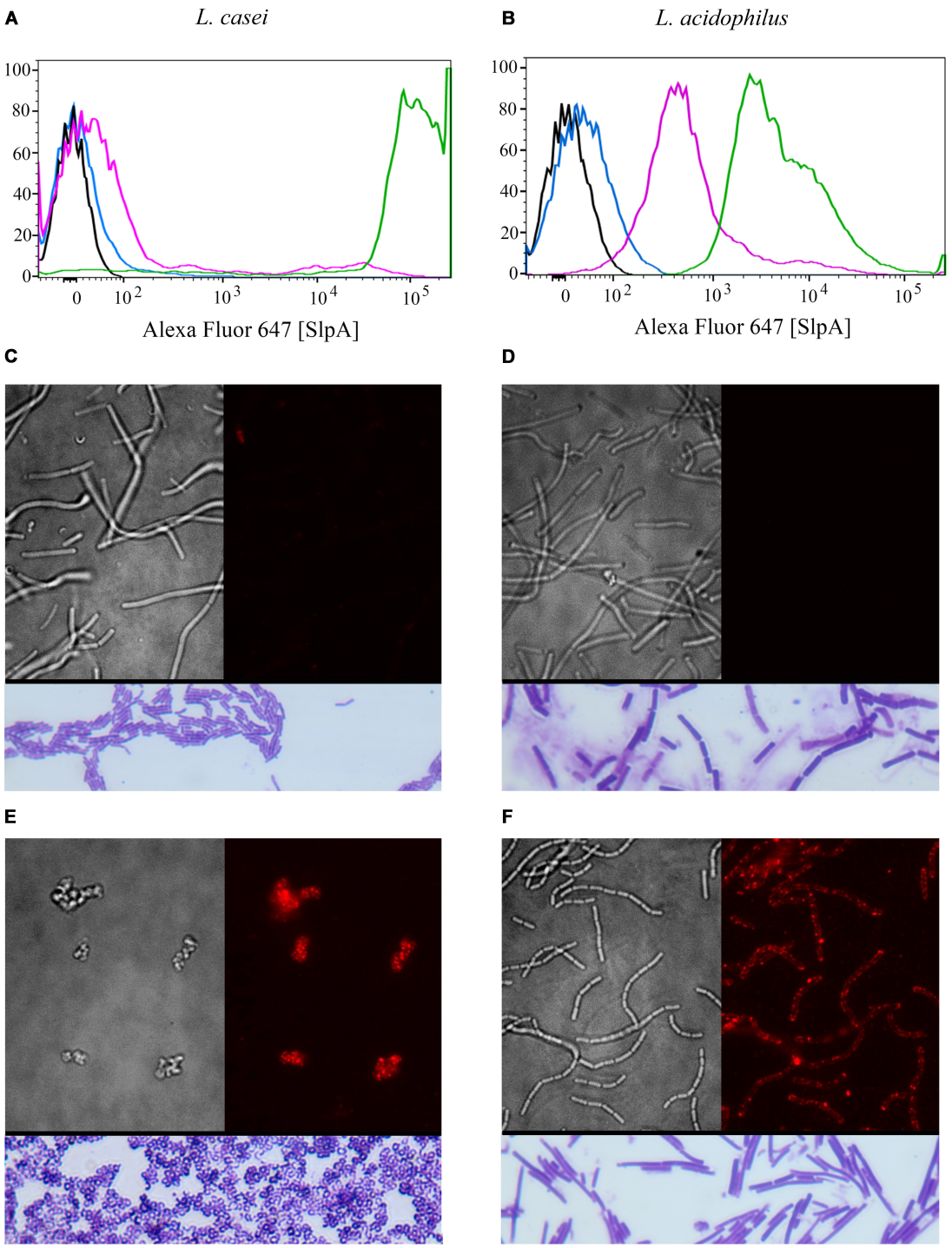


FIGURE 2 | Syn-LAB 2.0 and Syn-LAB 2.1 surface-display chimeric SlpA. **(A,B)** Fluorescence-activating cell sorting analyses. **(A)** Black, unstained *L. casei* parent strain; Blue, unstained Syn-LAB 2.0; Magenta, SlpA-stained *L. casei* parent strain; Green, SlpA-stained Syn-LAB 2.0 (median fluorescence > 100,000). *L. casei* does not have a classic S-layer, therefore, Syn-LAB shift is unique. **(B)** Black = unstained *L. acidophilus* parent strain; Blue, unstained Syn-LAB 2.1; Magenta, SlpA-stained *L. acidophilus* parent strain; Green, SlpA-stained Syn-LAB 2.1 (median fluorescence > 4,000). *L. acidophilus* has a native S-layer, therefore, chimeric SlpA is detected as a discrete, strong fluorescence shift. **(C–F)** microscopy; **(C)** brightfield image, *L. casei* parent strain with minimal detectable SlpA fluorescence; **(D)** brightfield image, *L. acidophilus* parent strain with undetectable SlpA fluorescence; **(E)** immunofluorescence, Syn-LAB 2.0 with intense, punctate, SlpA staining, and **(F)** immunofluorescence, Syn-LAB 2.1 with intense SlpA staining. All strains were probed with a *C. difficile* -specific anti-SlpA serum. Images are representative of at least 20 fields and > 1000 bacteria visualized. All images were visualized with a high-resolution DeltaVision deconvolution microscope. Gram's stained bacteria are shown in rectangles below each of **(C–F)**.

purple/blue in the presence of the corresponding carbohydrates. Gram's staining revealed that in contrast to the rod-shaped morphology of the parent *L. casei* strain, Syn-LAB 2.0 cells were shorter and curved (**Figures 2C,E**). Syn-LAB 2.1 bacteria were indistinguishable from the parent *L. acidophilus* strain (**Figures 2D,F**).

One isolate each of Syn-LAB 2.0 and Syn-LAB 2.1 as well as the respective parent *Lactobacillus* sp strains were used with appropriate antibiotic selection for the *in vitro* studies presented below. For *in vivo* studies, Syn-LAB 2.0, Syn-LAB 2.1, a combination thereof, or "empty vector" harboring *Lactobacillus* sp. strains were tested; no selection antibiotics were used in animals.

Syn-LAB Strains Display Chimeric *C. difficile* SlpA

SlpA chimera expression was confirmed via multiple methodologies for both engineered biologics (Syn-LAB 2.0 and Syn-LAB 2.1). First, flow cytometry was used to determine the degree of heterologous (chimeric) SlpA surface display. In actively growing cultures, almost 100% of Syn-LAB 2.0 bacteria displayed the *C. difficile* SlpA chimera (confirmed by fluorescence shifts in the engineered isolate compared to the parent strains (**Figure 2A**). Similar results were obtained for Syn-LAB 2.1 (**Figure 2B**), confirming robust SlpA display in that biologic as well.

Second, chimeric SlpA expression and display was visualized via immunofluorescence using anti-*C. difficile* SlpA antiserum (Merrigan et al., 2013). In contrast to the parent *L. casei* strain (**Figure 2C**), the engineered Syn-LAB 2.0 derivative revealed dense SlpA staining (**Figure 2E**). Similarly, Syn-LAB 2.1 (**Figure 2F**), but not the parent *L. acidophilus* strain (**Figure 2D**), exhibited intense and punctate surface SlpA staining. Staining was specific since no signal was detected on either Syn-LAB strain with pre-immune serum (not shown).

Chimeric *C. difficile* SlpA Is Incorporated Into the Lactic Acid Bacterial Cell Surface

We also confirmed that the *C. difficile*-specific SlpA visualized in **Figure 2** above was due to incorporation of the chimeric protein into the LAB cell wall. Sheared total Surface-Layer (S-layer) proteins from Syn-LAB 2.0 and Syn-LAB 2.1, and the isogenic parent strains (Merrigan et al., 2013), were separated via agarose gel electrophoresis (**Figure 3A**), and immunoblotted using the anti-SlpA antiserum. Both synthetic biologics, but not the isogenic parent strains, displayed altered total S-layer profiles, and a discrete unique band, appropriate to the expected size of the chimera, (**Figure 3B**). Mass spectrometry confirmed the presence of chimeric SlpA sequences (not shown). Due to the polyclonal nature of the antiserum other non-specifically reacting bands were also observed in the parent strains.

Chimeric SlpA Expression Preserves Epithelial Barrier Function

We performed a series of studies to rule out potential adverse effects of the engineered strains on intestinal epithelial cell health and function. First, potential impact of Syn-LAB strains on intestinal epithelial barrier function was assessed via *trans*-epithelial electrical resistance (TEER) measurements. Unexpectedly, the parent *L. casei* strain decreased TEER over a 7-h period, with changes becoming consistently apparent as early as 3 h post-application (**Figure 4A**). In contrast, addition of Syn-LAB 2.0 did not significantly alter TEER relative to mock-treated cells. Similarly, Syn-LAB 2.1, or the parent *L. acidophilus* strain, had no impact on the TEER of C_{2BBe} cells (**Figure 4B**). This suggests that Syn-LAB 2.0 and Syn-LAB2.1 do not disrupt host epithelial barrier function.

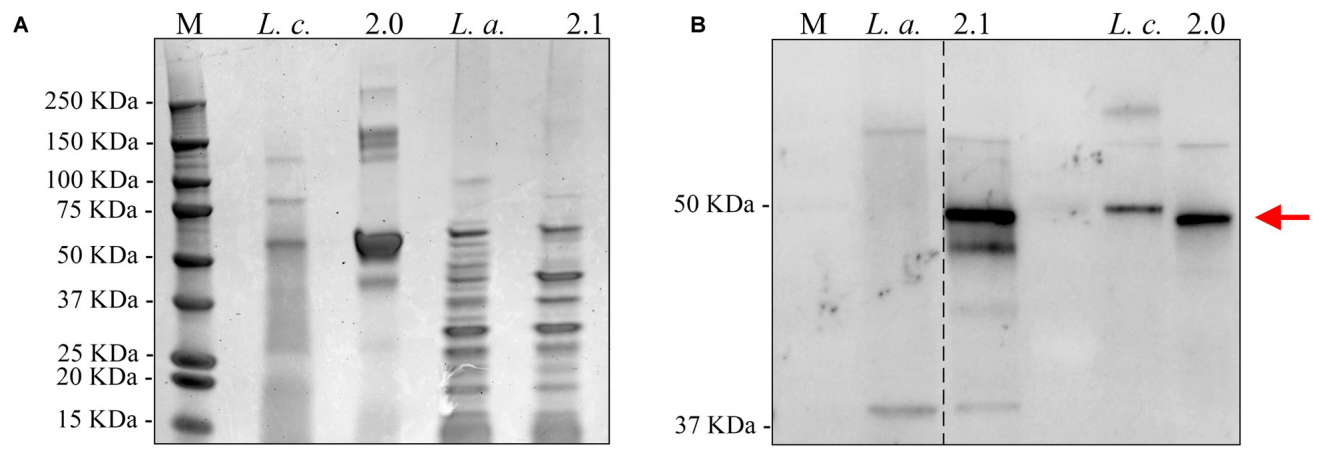


FIGURE 3 | Chimeric *C. difficile* SlpA is incorporated into the lactic acid bacterial (LAB) cell wall. **(A)** Sheared total S-Layer Proteins from the *L. casei* parent strain (Lc) or Syn-LAB 2.0; and the *L. acidophilus* parent strain (La), or Syn-LAB 2.1. **(B)** Immunoblot probing the total Surface Layer Proteins of parent and Syn-LAB strains with antiserum specific to *C. difficile* SlpA. The red arrow points to unique, expected-size bands present only in Syn-LAB 2.0 and Syn-LAB 2.1 respectively. The dotted line indicates that the original immunoblot was edited to remove irrelevant lanes.

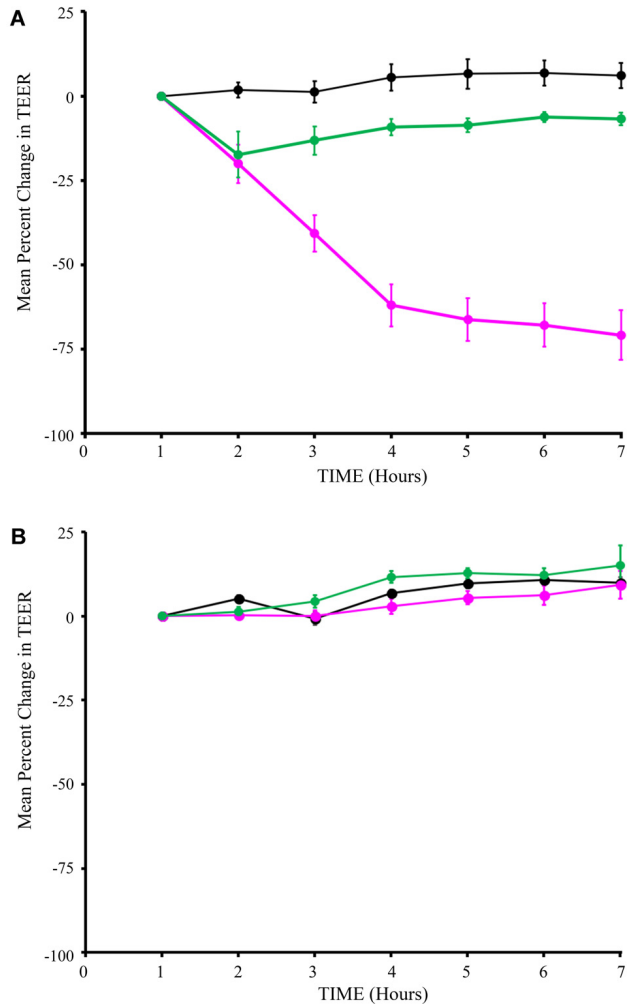


FIGURE 4 | Syn-LAB 2.0 preserves epithelial barrier function. Transepithelial electrical resistance (TEER) measurements of $C2_{BBe}$ monolayers that were mock-treated (black line), or exposed to the parent *L. casei* strain (green line), or Syn-LAB 2.0 (magenta line; **A**), or parent *L. acidophilus* strain (green line), or Syn-LAB 2.1 (magenta line; **B**), for 8 h. Experiments were performed in at least three biological replicates. Host cells were treated with 100 bacteria per cell (MOI = 100).

Chimeric SlpA Display Does Not Compromise Host Cell Viability

Next, we assessed the impact of Syn-LAB on the survival of host intestinal epithelial cells. $C2_{BBe}$ cells were grown to confluence, and either mock-treated, or treated with *L. casei, L. acidophilus,* or the respective Syn-LAB derivatives for up to 8 h. Cell death was continuously monitored via propidium iodide (PI) uptake, a DNA-intercalating dye that only enters dying cells with compromised cell membranes. *L. casei, L. acidophilus,* and the respective chimera-expressing Syn-LAB derivatives did not significantly impact epithelial cell viability relative to mock-treated cells (**Figures 5A,B**).

Syn-LABs Are Safe and Tolerable in Multiple Animal Models, and Robustly Colonize the Mammalian Gastrointestinal Tract (GIT)

PCR- and microbiologically verified pure cultures of Syn-LAB strain 2.0 was used for these studies, and prepared for *in vivo* dosing at $\sim 10^8$ bacteria per 200 μL volume of PBS. To conserve animals, the shedding/colonization assessments were performed as pilot studies. For broad assessment of Syn-LAB shedding, two animal models (Golden Syrian hamsters and neonatal piglets) were utilized, with each model testing one of the two Syn-LAB strains respectively.

Golden Syrian hamsters received either no treatment (control), or a single dose of the broad-spectrum antibiotic

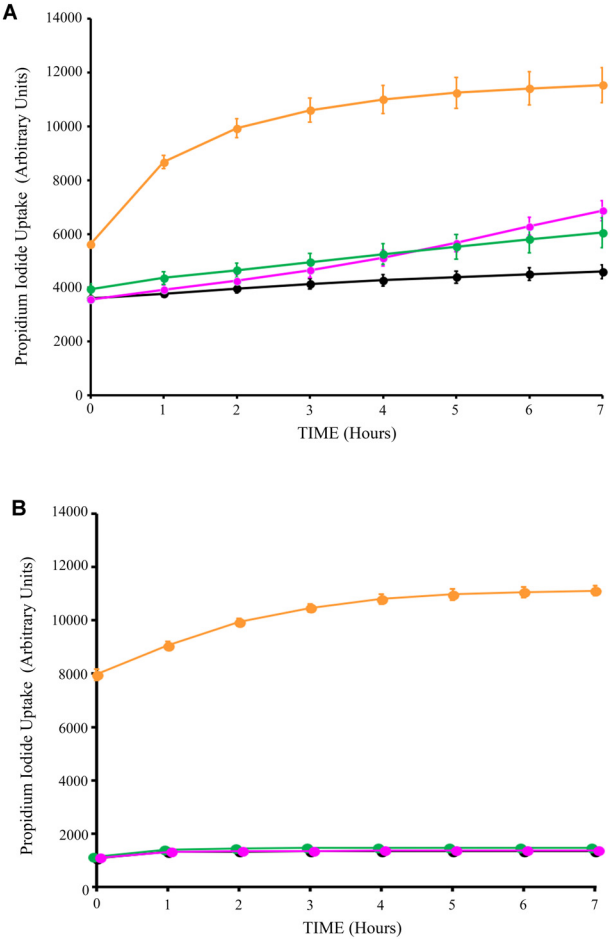


FIGURE 5 | Lactic acid bacteria (LAB) treatment does not impact host-cell viability. Propidium iodide uptake assays of $C2_{BBe}$ monolayers mock-treated (black line), or exposed to the parent *L. casei* strain (green line), or Syn-LAB 2.0 (magenta line; **A**), or parent *L. acidophilus* strain (green line), or Syn-LAB 2.1 (magenta line; **B**), for 8 h. Orange line shows methanol-treated monolayers representative of maximal cell death. Experiments were performed in at least three biological replicates. Host cells were treated with 100 bacteria per cell (MOI = 100).

clindamycin, followed by 6 daily doses of the *L. casei*-based Syn-LAB 2.0 (antibiotic "pre-treatment" group), or 3 days of Syn-LAB 2.0, followed by a single dose of oral clindamycin (as above), followed by another 3 days of Syn-LAB 2.0 (antibiotic "mid-cycle treatment" group). Syn-LAB treated hamsters started shedding the biologic on Day 1 post-administration; this continued until the end of the study (similar studies were performed for Syn-LAB 2.1, with identical observations; not shown). Hamsters that received clindamycin on Day 4 post-Syn-LAB showed no evidence of the biologic on Day 5, confirming the *in vivo* susceptibility of Syn-LAB 2.0 to standard antimicrobial therapy (**Figure 6A**). However, when Syn-LAB administration was restarted, shedding resumed at a magnitude similar to that observed prior to antimicrobial therapy (**Figure 6A**). Importantly, all hamsters were similarly colonized, with Syn-LAB fecal titers reaching, or exceeding, 10^5 colony forming units/gram stool on Day 3 post-treatment (**Figure 6B**). Immunofluorescence studies of colonic tissues harvested post-necropsy revealed dense luminal staining only from Syn-LAB 2.0 treated hamsters (**Figure 6C**, right panel) as compared with mock-treated animals (**Figure 6C**, left panel), confirming *C. difficile* SlpA expression in the hamster gastrointestinal tract. Finally, Syn-LAB 2.0 was avidly consumed by all hamsters, with no requirement for a pre- or post-dosing sweetened electrolyte "chaser." Safety and tolerability were also confirmed via lack of any adverse effects in any Syn-LAB-treated hamsters, as well as appropriate activity and alertness throughout the study.

Continuous Syn-LAB Administration Induces an Anti-*C. difficile* SlpA Immune Response

While the primary goal was to design biologic agents that could competitively occupy *C. difficile* attachment sites in the gut, we also explored the possibility of an anti-SlpA immune response following long-term Syn-LAB administration. Golden Syrian hamsters were continuously administered Syn-LAB 2.0 as a once-daily 10^8 CFU dose for 55 days. Hamsters shed the biologic consistently throughout the process confirming that they were appropriately colonized. Age- and weight-matched control hamsters received no treatment. At the end of the study, hamsters were humanely euthanized, whole blood collected, and immunoblot-based analyses performed to assess anti-Syn-LAB immune response. Serum from Syn-LAB 2.0-treated hamsters (**Figure 7A**, top right panel), but not from mock-treated animals (**Figure 7A**, top left panel), detected *C. difficile* strain 630 SlpA in a dose-dependent manner. Presence of Slp proteins in the corresponding membranes was verified by re-probing the blots with a SlpA-specific antiserum previously generated in our laboratory (**Figure 7A**, lower panels). Finally, the same experiments were performed using Slp preparations from clinically-relevant isolates of diverse *C. difficile* ribotypes (012, 017, 020, 027, 078). Reactivity was observed only when serum from Syn-LAB-treated hamsters was used (**Figure 7B**). This suggested that the Syn-LAB SlpA moiety elicited a cross-reactive immune response (recognition of non-cognate *C. difficile* SlpA).

Syn-LABs Protect Syrian Golden Hamsters From *C. difficile*-Induced Death

Since single-species probiotics are thought to have limited ability to protect against CDI (Wullt et al., 2003; Vernaya et al., 2017), we used a combination of Syn-LAB 2.0 and Syn-LAB 2.1 in hamster protection studies. A mixed culture of the biologics (10^8 CFU total) was administered to antibiotic-sensitized Golden Syrian hamsters either as a fixed dose (FD) formulation (6 doses) or as a continuous dose (CD) formulation (3 days prior to clindamycin until the end of the study). Syn-LAB-treated hamsters were compared to those receiving *C. difficile* alone, or those administered LAB containing the empty vector. Challenge studies used a high inoculum (∼1000 spores) of *C. difficile* strain 630 [a virulent, outbreak-associated isolate (Merrigan et al., 2013)].

Fixed dosing of the Syn-LAB combination significantly delayed death of hamsters compared to mock-treated animals, as well as those administered the empty-vector-harboring strains (**Figure 8A**). Continuous administration of Syn-LABs afforded statistically significant protection against CDI throughout the course of 12 days of infection (**Figure 8B**). Specifically, and as compared with untreated hamsters, protection was highly significant at multiple time points during the infection course ($p = 0.0091$, $p = 0.0014$, $p = 0.0005$, $p = 0.0005$ at 6, 8, 11 and 12 days post-infection respectively). This was in contrast to the protection afforded when hamsters were administered the parent LAB strain harboring the empty vector ($p = 0.1147$, $p = 0.0147$, $p = 0.0147$, $p = 0.0147$ on Days 6, 8, 11, and 12 post-infection respectively; 99% confidence interval for significance). Additionally, parent strain-treated hamsters succumbed to disease earlier in the infectious course, and were more often found moribund, with symptoms consistent with fulminant CDI (profound wet-tail, lethargy, sternal recumbency and cecal hemorrhage).

Re-administration of clindamycin to Syn-LAB-treated, *C. difficile*-challenged hamsters 14 days post infection did not result in disease or mortality (not shown). This suggested that Syn-LAB-mediated colonization resistance also ablated *C. difficile* persistence. Taken together, Syn-LAB administration was highly protective in the hamster model described above.

Fixed-Dose Syn-LAB Administration Protects Neonatal Piglets From *C. difficile*-Induced Diarrhea

Preliminary neonatal piglet studies used healthy, newborn animals treated with vancomycin on Day 4 post-farrowing to ensure elimination of any carryover *C. difficile* bacteria from the farm. Control piglets were given PBS followed by *C. difficile* challenge, whereas treated piglets were administered three 10^8 CFU doses of the fast-growing Syn-LAB 2.1 strain over 24 h. Syn-LAB 2.1 was detected as early as 24 h after the first dose, and shedding continued until the end of the study (not shown).

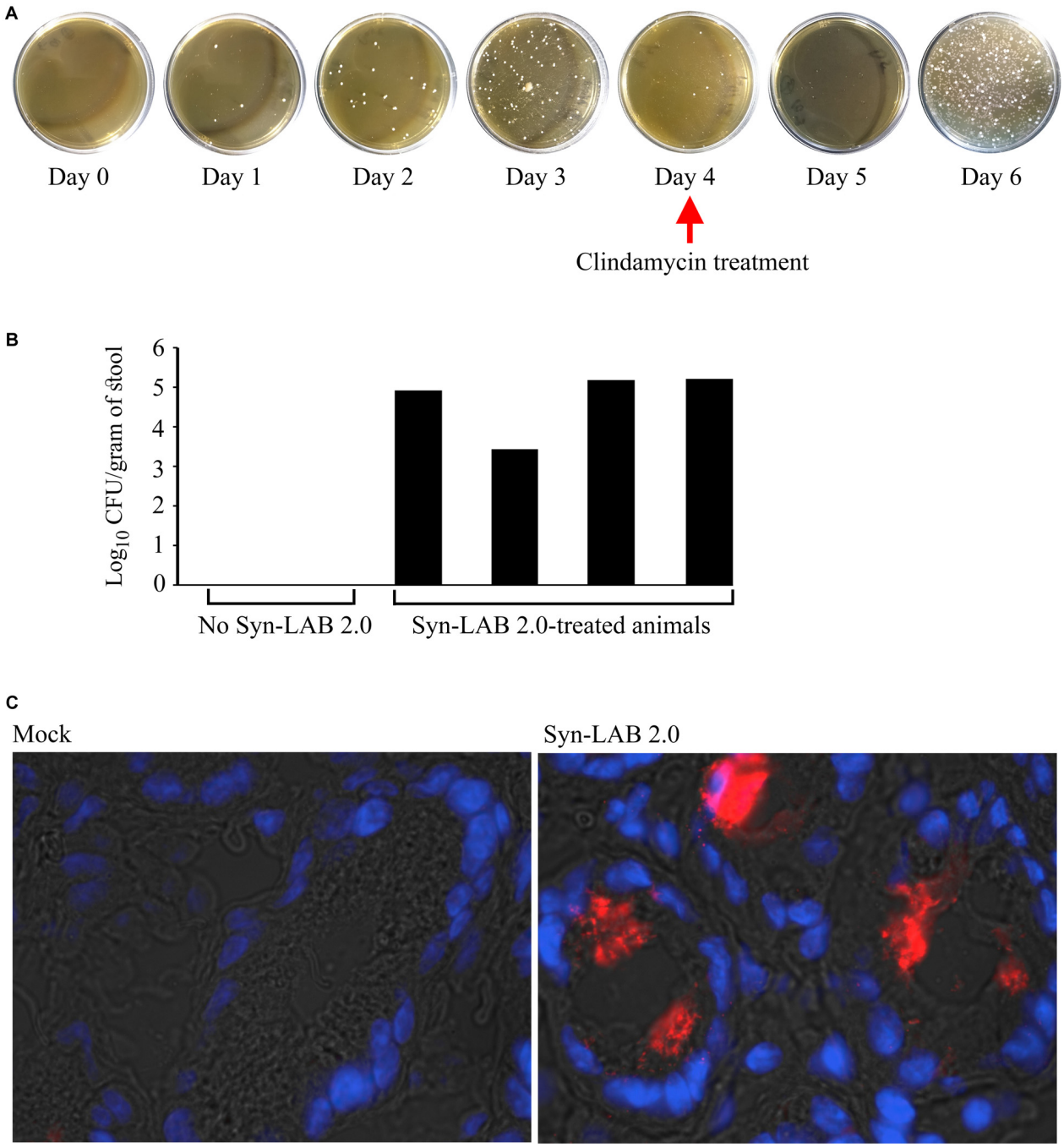


FIGURE 6 | Syn-LAB colonization of Golden Syrian Hamsters. **(A)** Syn-LAB 2.0 colonizes the hamster gastrointestinal tract, and is shed as early as 1 day post-administration. Hamster stool pellets from animals continuously administered Syn-LAB 2.0 at 10^8CFU/day cultured on MRS-chloramphenicol. Antibiotic treatment on Day 4 ablates Syn-LAB 2.0 shedding only for 1 day, followed by continued detection from Day 5 onward. Data are representative of 8 infected animals. **(B)** Syn-LAB 2.0 shedding is comparable in all treated animals (black bars), but not detectable in control, untreated, animals. **(C)** immunofluorescence analysis using anti-*C. difficile* SlpA serum reveals intense staining in the colonic lumen of Syn-LAB 2.0-fed animals (right), but not mock-treated animals (left). Images are representative at least 10 fields visualized per section.

In this model, CDI [1000 spores for piglets (Steele et al., 2010)], resulted in profuse diarrhea (**Figure 9A**, left panel, stool score of 1). Diarrheic symptoms in these piglets continued unabated for at least 3 days, at which time the accumulated dehydration and inappetance criteria necessitated euthanasia. Microscopic examination of colonic tissues from infected piglets revealed gross hemorrhage with an abundance of inflammatory infiltrates (**Figure 9B**, middle panel). However, piglets that received a 1-day

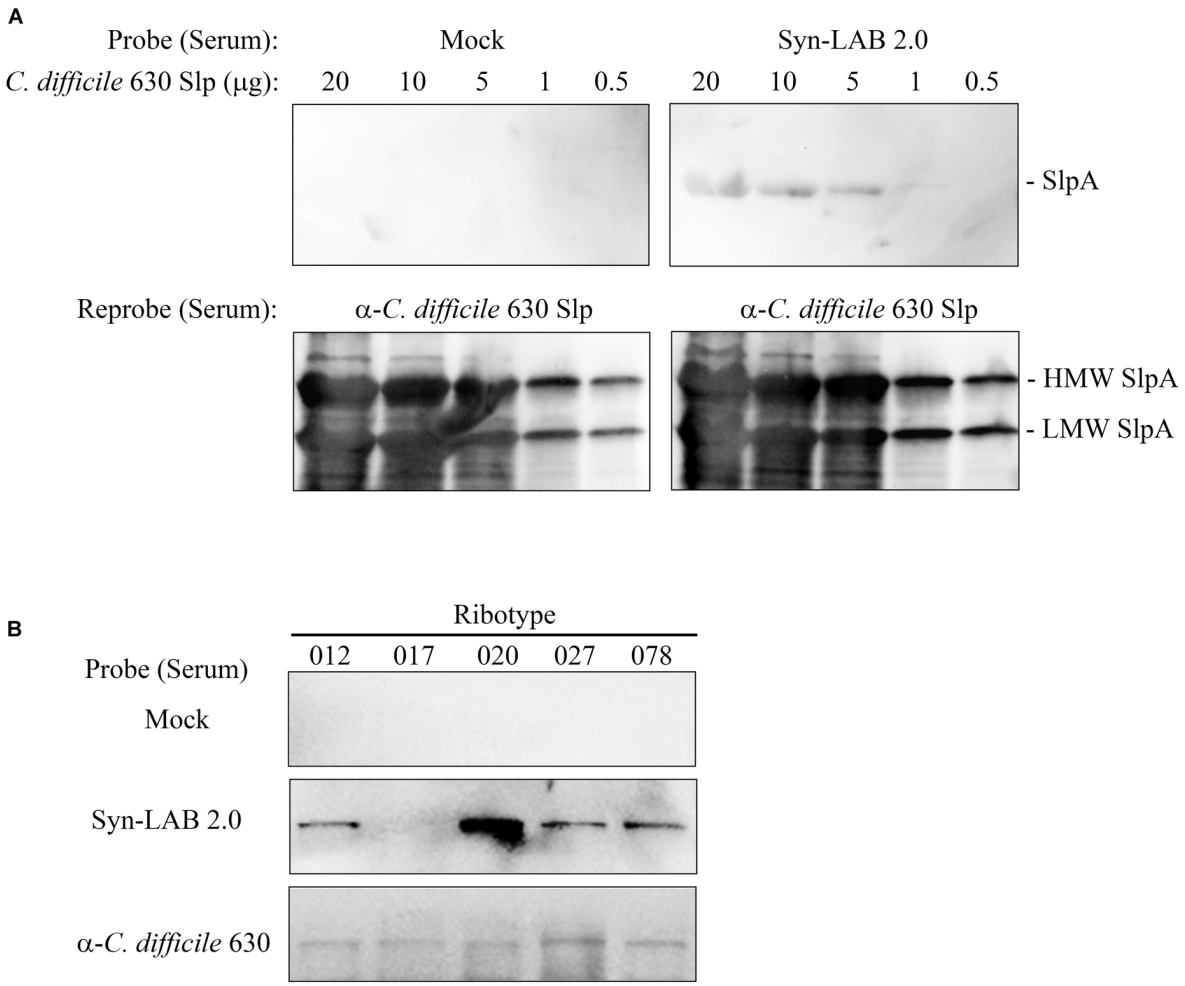


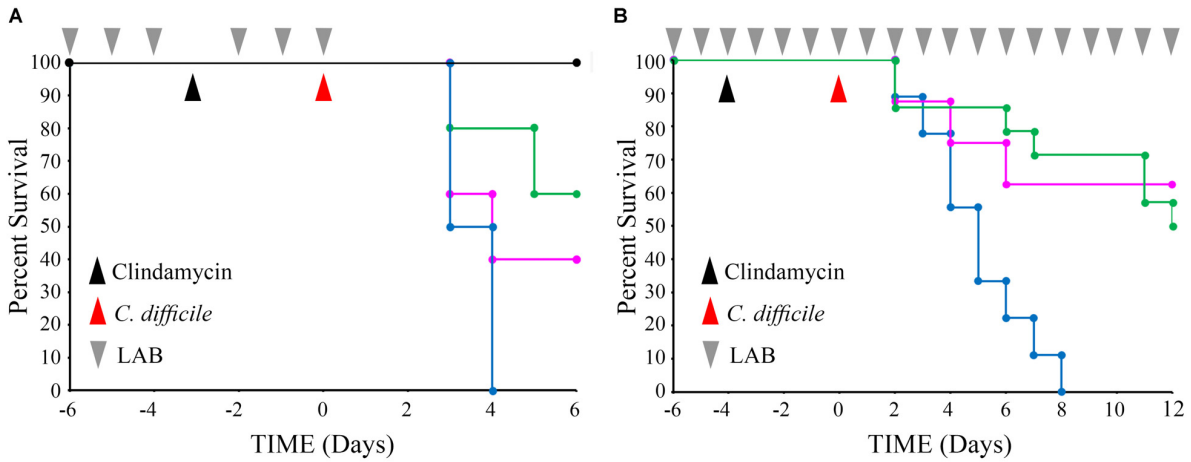
FIGURE 7 | Syn-LAB 2.0 elicits an anti-*C. difficile* SlpA immune response. **(A)** Top panels, dose-response immunoblots of S-layer proteins (20 to 0.5 μg) from *C. difficile* strain 630 probed with serum from a Syn-LAB 2.0-treated animal (right), or serum from an untreated, age- and weight-matched hamster (left). Bottom panels, to verify efficient separation and transfer of the S-layer proteins, the membranes were stripped and re-probed with a polyclonal *C. difficile* anti-SlpA antiserum. Both *C. difficile* SlpA subunits were detected (arrows). **(B)** S-layer proteins from *C. difficile* clinical isolates of diverse ribotypes probed with serum from an untreated animal (upper row), a Syn-LAB 2.0-treated animal (middle row), or with anti-*C. difficile* strain 630 SlpA serum (bottom row).

administration of Syn-LAB 2.1 had well-formed stool (Stool score of 3–4; **Figure 9A**, right panel), as well as normal activity and appetite. Colonic tissue from these animals showed markedly less hemorrhage and inflammatory damage compared to those from piglets with CDI alone (**Figure 9B**, right panel).

DISCUSSION

With the emergence of outbreak-associated strains in the past decade, CDI has become a problem of considerable magnitude in terms of human and economic costs (Viswanathan et al., 2010). The protection offered by a healthy microbiota, known as colonization resistance, is the most effective foil against CDI (Seekatz et al., 2018). Although antibiotic-mediated dysbiosis is the most typical precipitating factor for CDI, traditional therapeutic options rely on administration of more antibiotics (McDonald et al., 2018). Apart from concerns of increased antibiotic resistance in *C. difficile*, this approach aggravates intestinal dysbiosis and, in a subset of infected individuals, results in recurrent disease (Viswanathan et al., 2010). Therefore, strategies that exploit colonization resistance to prevent or treat CDI can not only be effective in mitigating disease, but also address the underlying dysbiosis.

Two broad therapeutic approaches that exploit colonization resistance are fecal microbiota transplantation (FMT) and probiotic administration. In randomized trials, FMT efficacy ranges from ∼50% to 90% based on delivery and number of infusions (van Nood et al., 2013; Youngster et al., 2014; Cammarota et al., 2015; Kelly et al., 2016; Lee et al., 2016), but this procedure is logistically challenging and could pose undefined risks to patients (Wang et al., 2016; Gardiner et al., 2018); as such, it is recommended only for patients that repeatedly fail antibiotic therapy (at least 3 CDI episodes; IDSA-SHEA



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	Animals Remaining				
Group	Day 0	Day 6	Day 8	Day 11	Day 12
C. difficile only	9	2	0	0	0
C. difficile + Empty Vector	8	5	5	5	5
C. difficile + Syn-LAB	14	11	10	8	7

	P-value (Log-Rank Test)			
Group Comparison	Day 6	Day 8	Day 11	Day 12
C. difficile + Empty Vector Vs. *C. difficile* only	0.1147	0.0147	0.0147	0.0147
C. difficile + Syn-LAB Vs. *C. difficile* only	0.0091	0.0014	0.0005	0.0005
C. difficile + Syn-LAB Vs. *C. difficile* + Empty Vector	0.4396	0.7603	0.9393	0.7380

FIGURE 8 | Syn-LAB biologics protect hamsters from lethal CDI. **(A,B)** Co-administration of Syn-LAB 2.0 and 2.1 protects Golden Syrian hamsters from CDI. **(A)**, pilot study; Kaplan–Meier survival plot. Pre-treatment with Syn-LABs (6 once-daily doses of 1×10^{10} CFU each) delays death of hamsters infected with 1000 *C. difficile* spores. Black line, untreated, uninfected animals; blue line, *C. difficile*-infected animals; magenta line, animals treated with parent LAB strains harboring empty vector, and then infected; green line, animals treated with Syn-Lab 2.0+2.1 and then infected. **(B)**, powered study, Kaplan–Meier survival plot. Continuous Syn-LAB 2.0+2.1 dosing (pre- and post-infection) prevents death of *C. difficile*-infected hamsters. blue line, *C. difficile*-infected animals; magenta line = animals treated with parent LAB strains harboring empty vector, and then infected; green line = animals treated with Syn-Lab 2.0+2.1 and then infected. **(C)** Group comparisons and statistical test results. Number of surviving animals in powered study (top), and Log-Rank tests (bottom). $p \leq 0.01$ = significant.

guidelines (McDonald et al., 2018). While probiotics are more palatable and pose fewer risks, they show variable efficacy in treating CDI (Rezaie and Pimentel, 2014; McFarland, 2015; Barker et al., 2017; Goldenberg et al., 2017; Alberda et al., 2018). Some studies have shown probiotic efficacy when used in patients with no CDI history, but differences in formulation, dose, dosing duration and species composition preclude strong conclusions being drawn in favor of probiotics as CDI interventions. Indeed, the latest IDSA-SHEA recommendations for CDI intervention do not mention probiotics as a treatment option, and no recommendation is made for the agents in primary disease prevention (McDonald et al., 2018). Our goal was to develop a biologic agent for colonization resistance against CDI with consistent and robust efficacy against CDI, but with a safety profile comparable to extensively used probiotics. We, therefore, sought to engineer the "Generally Regarded as Safe" (GRAS) organisms *Lactobacillus casei* and *Lactobacillus acidophilus* to express *C. difficile* surface adhesins and, thereby, competitively exclude the pathogen from intestinal surfaces.

Lactobacilli can be extraordinarily recalcitrant to manipulation; this is both an advantage and a liability. The difficulties encountered in introducing or extracting DNA from the various species portends well for the use of the organisms in probiotic preparations due to reduced risk of horizontal acquisition of antibiotic resistance genes from endogenous microbiota. However, laboratory manipulation poses unique challenges. Indeed, only electroporation with high amounts ($\geq$10 μg) of DNA, and a strain-specific optimized protocol, is the recommended method to transform *Lactobacillus* sp; despite this, reported efficiencies may be as low as 1 transformant/μg DNA (Vogel and Ehrmann, 1996). On balance, however, *Lactobacillus casei* and *Lactobacillus acidophilus* offer unique advantages that can be exploited for CDI treatment. *L. casei* can suppress the inflammatory cytokines produced in response to CDI (Boonma et al., 2014), upregulate mucin gene expression (Mattar et al., 2002), and also appears to confer human subjects some protection from CDI when administered as a fermented drink (Wong et al., 2014; Alberda et al., 2018).

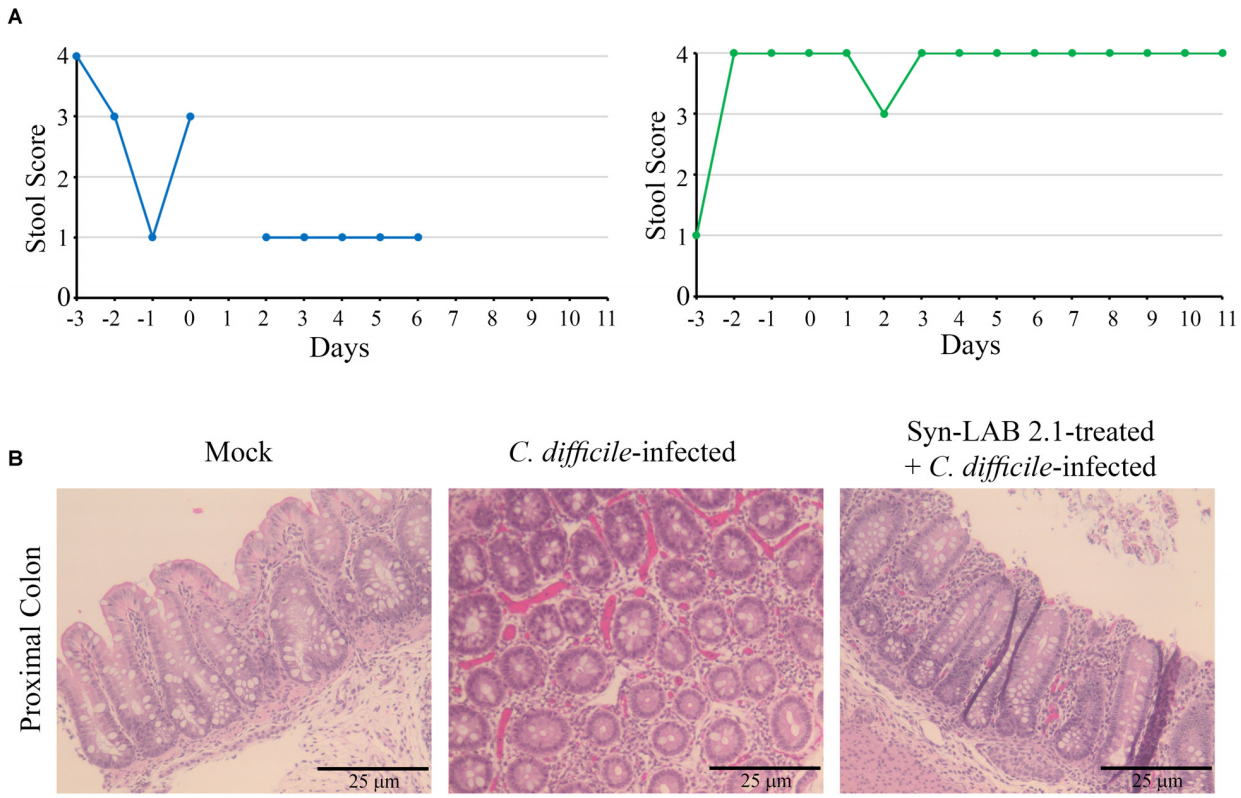

FIGURE 9 | Syn-LAB 2.1 protects piglets from CDI diarrhea. Pilot, non-lethal CDI model. **(A)** Stool consistency scoring; 1 = diarrheic, 4 = fully formed. Left, *C. difficile*-infected animal, with consistently low stool scores indicating unremitting diarrhea. Right, Syn-LAB 2.1 treated + *C. difficile*-infected animal, with consistently high stool score, indicating no diarrhea. The example shown is representative of 3 animals studied. The animal in the left panel was euthanized after the last time-point shown due to unremitting diarrhea, increasing dehydration and inappetence. Similar results for the other two animals. All infected animals in the group received 1000 spores of *C. difficile*. **(B)** microscopic examination of proximal colon tissues from piglets. Left, hematoxylin-eosin staining of tissue from uninfected animal showing normal epithelium, little/no inflammatory infiltrate and no overt damage or necrosis. Middle, *C. difficile*-infected animal, revealing gross hemorrhage with an abundance of inflammatory infiltrates. Right, Syn-LAB 2.1-treated and *C. difficile*-infected animal tissue showing marked reduction in both overt hemorrhage and inflammation.

L. acidophilus has been shown to decrease *C. difficile* toxin gene expression and also protect animals in a murine CDI model (Yun et al., 2014). However, data regarding the strain-specific benefits of these organisms, or their consistently beneficial use in diverse patient cohorts, are scarce. For Syn-LAB engineering, we used pure, genome-sequenced, antibiotic sensitive strains of both genera. Lactobacilli are notoriously recalcitrant to taxonomic classification, and phenotypic or biochemical identification, and therefore need to be subjected to extensive molecular identification to confirm species purity (Pal et al., 2012). The Syn-LAB 2.0 antecedent strain is morphologically and biochemically distinguishable from the Syn-LAB 2.1 parent, allowing for clear discrimination of the strains.

The CD/LAB chimeric SlpA was robustly expressed in both *Lactobacillus* species, integrated into the cell wall, and displayed on the surface; thus, the surface display of the chimera was not hindered by presence of the native LAB S-layer. We assessed Syn-LAB dosing and maintenance, and its ability to protect against a high dose of *C. difficile* spore infection (> 1000 spores) in the lethal hamster model of infection [a spore dose of ∼100 causes 100% lethality (Sattar et al., 2015)]. Continuous Syn-LAB dosing was easily achieved in hamsters, and the bacteria were readily detected in high numbers in the stool as early as 1 day post-administration. With fixed dosing, however, the biologics gradually declined in numbers in the stool, and were below the level of detection within 5 days post-administration (not shown). Such tunable maintenance of the biologics in the gut would be sparing of the endogenous microbiota, whose reestablishment after antibiotic insult is critical to CDI relief.

Even with fixed, pre-challenge dosing (6 doses), the engineered Syn-LAB 2.0 strain afforded protection against CDI. With continuous dosing of the biologic agent, however, there was significant protection against disease as well as mortality, even in the absence of any plasmid maintenance antibiotics (none were used in our animal studies). Treatment with the parent LAB strains was partially protective with continuous dosing compared to no treatment at all; this was not statistically significant compared to untreated animals, and was more variable, correlating with observations in human clinical studies where probiotic use does not consistently protect against CDI (Mills et al., 2018).

Beyond colonization resistance, the elicitation of anti-*C. difficile* SlpA antibody response in Syn-LAB-treated hamsters is noteworthy. In previous studies, recombinant SlpA-vaccinated mice exhibited a modest decrease in subsequent fecal *C. difficile* shedding, and an anti-SlpA response afforded partial protection against CDI (Biazzo et al., 2013; Bruxelle et al., 2016). Thus, Syn-LAB strains could afford long-term protection from new- or re-infection with diverse *C. difficile* strains in the community or healthcare setting. The use of these targeted biologics, therefore, via a once-daily oral administration as we tested herein, is likely to be suitable for multiple hosts.

Unlike the lethal hamster model, piglets are natural hosts to CDI, and display symptoms similar to human infections. Recent studies have highlighted CDI burden in agriculture, and its impact on the swine industry (Grzeskowiak et al., 2016; Stein et al., 2017; Kim et al., 2018). Our preliminary studies in this model are promising: as with the hamster studies, Syn-LAB was delivered and maintained in the piglets with ease, with the biologic being shed in the stool consistently. Even with a single-day FD regimen, the piglets were protected against CDI-induced diarrhea, in contrast to animals given *C. difficile* alone.

CONCLUSION

Individuals (or animals) who are not appropriate candidates for anti-CDI immunization, those anticipating extended-duration antibiotic treatment, or those in long-term care facilities (LTCFs) where the risk of acquiring CDI is high may benefit from Syn-LAB-type agents. Further, asymptomatic carriage of *C. difficile* is considered to be a major factor in pre-disposing patients to active CDI (Blixt et al., 2017; Caroff et al., 2017), and Syn-LAB administration may substantially reduce this risk.

LIMITATIONS OF THIS STUDY

The studies as presented support Syn-LAB biologics as powerful new tools to prevent CDI in multiple mammalian systems. However, we recognize limitations that will need to be addressed by fully powered studies prior to completion of Syn-LAB pre-clinical testing. First, Syn-LAB efficacy will need to be tested against an even wider panel of recent *C. difficile* clinical isolates than those shown in **Figure 7**. However, the strong serum reactivity from Syn-LAB-treated animals against diverse, clinically relevant *C. difficile* ribotypes is encouraging. Second, Syn-LAB 2.0 and 2.1 may differ in their individual propensities to protect against CDI, and this needs to be investigated further. Third, we need to evaluate if the anti-SlpA antibody response results in protective immunity against *C. difficile* challenge, and parse this from colonization resistance provided by the biologic itself. Finally, the ability of Syn-LABs as a therapeutic (and not just a prophylactic as presented herein) remains to be tested – and this will involve efforts to determine biologic efficacy at different time-points post-infection. These limitations notwithstanding, our studies show strong evidence that the engineered Syn-LAB strains have considerable potential as primary or adjunct therapeutic agents against CDIs in multiple mammalian systems.

AUTHOR CONTRIBUTIONS

GV and VV conceptualized and funded these studies, wrote the manuscript, and provided full project oversight. JL, JK, MM, and JLR designed, performed, optimized, and interpreted the experiments. CA, FA, AC, RC-W, AM, RCM, RM, and SR performed, supported and fully participated in all animal studies as well as some *in vitro* confirmation studies (FA and RM). All the authors read the manuscript and provided feedback.

FUNDING

This research project was supported by the National Institutes of Health (GV; AI121590). Work in the Vedantam laboratory is also supported by the US Department of Veterans Affairs (1I01BX001183-01) and the USDA CSREES Hatch Program (ARZT-570410-A-02-139; GV). The pilot piglet studies reported herein were supported by an Asset Development Award from Tech Launch Arizona.

ACKNOWLEDGMENTS

Special thanks to Dr. Todd Klaenhammer for the kind gift of the pTRK848 plasmid. Many thanks to all the current and former members of the Vedantam and Viswanathan laboratories, specifically Michele Chu and Andrea Treptow. The help and support of the University of Arizona Cancer Center ARL Cytometry Core Facility (Grant-CA 023074) is gratefully acknowledged. We also thank Dr. Tod McCauley for helpful discussions.

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